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*DIFFERENTIAL MORTALITY AMONG THE SEXES IN CHILDHOOD AND ITS POSSIBLE SIGNIFICANCE IN HUMAN EVOLUTION**

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Communicated February 18, 1963

During a study of agricultural practices[1, 2] of Ladino and Indian families of the region around Lake Petén, El Petén, Guatemala, a rather peculiar demographic situation was noticed among the Indians. In contrast with Ladino families living in the same region and frequently in the same communities, it appeared that events were occurring among Indians that resulted in a sex ratio by the fifteenth year of 1.8♂:1♀. The effect is not due to an abnormal neonate sex ratio, for the available figures indicate a ratio of less than unity. Cowgill and Hutchinson[3] concluded that the observed ratio in childhood is due to less careful nurture of female children between the ages of one and four years. In view of these results, it appeared to be of interest to determine just how widespread this phenomenon was in other populations, both in Central America and in the rest of the world.

Mode of Presentation.—If the sex ratio of individuals dying between specified ages is plotted against such ages, a characteristic pattern is revealed. Initially, there is a markedly greater mortality of boys, as is well known. This is partly due to the neonatal sex ratio being well over unity in practically every population reported;[4] but if the ratio is normalized to refer to a neonate population of equal numbers of both sexes, the effect still persists, and has been widely explained as due to hemizygous semilethals on the X-chromosome. It is obvious that with an increase in unselective infant mortality, the normalized ratio will fall asymptotically to unity, but cannot become less than one by this process.

After the first year the unnormalized ratio is systematically smaller in every population for which the figures are large enough to give significant ratios. In a considerable number of countries, none of which, however, are among the most industrialized and medically advanced, the unnormalized ratio falls below unity in the interval of one to four years of age. Subsequently, there is characteristically a rise in relative male mortality which in some populations becomes extreme, largely due to a high incidence of accidental deaths in early adolescence. Our concern in the present paper is with the nature of the fall in the ratio after the first year of life. In particular, we first inquire whether the normalized ratio, i.e., the ratio corrected to a sex ratio of unity in the individuals entering the period of two to five years,

TABLE 1

Country	—Total— Mortality 0–4 years as % live births (1)	Mortality <1 year as % live births (2)	Mortality 1–4 years as % live births (3)	Neonate sex ratio (4)	Uncorrected mortality ratio 1–4 (5)	Normalized mortality ratio 1–4 (6)
Netherlands	2.18	1.72	0.46	1.056	1.443	1.371
Scandinavia	2.43	2.02	0.41	1.055	1.380	1.316
Australia	2.49	2.05	0.44	1.050	1.311	1.240
England	2.57	2.25	0.32	1.059	1.360	1.292
Switzerland	2.68	2.22	0.46	1.055	1.408	1.343
U.S.A.	3.06	2.66	0.40	1.051	1.195	1.146
Scotland	3.11	2.77	0.34	1.053	1.113	1.064
Northern Ireland	3.18	2.81	0.37	1.075	1.354	1.268
New Zealand	3.24	2.63	0.61	1.061	1.391	1.320
France	3.26	2.71	0.55	1.045	1.186	1.144
Canada	3.48	3.02	0.46	1.058	1.241	1.182
Un. So. Africa: Whites	3.52	2.91	0.61	1.044	1.119	1.080
Eire	4.04	3.54	0.50	1.051	1.347	1.294
Germany, W.	4.15	3.64	0.51	1.067	1.249	1.182
Belgium	4.45	3.94	0.51	1.064	1.088	1.035
Japan	4.63	3.46	0.17	1.055	1.138	1.086
Algeria: Europeans	4.77	4.03	0.74	1.055	1.101	1.053
Bulgaria	5.37	5.22	0.15	1.057	1.038	0.993
Israel	5.51	4.44	1.07	1.066	0.825	0.775
Germany, E.	5.64	4.78	0.86	1.069	1.310	1.239
Uruguay	5.93	5.22	0.71	1.048	1.029	0.993
Italy	5.98	5.00	0.98	1.055	1.130	1.081
Spain	6.37	5.33	1.04	1.051	1.066	1.027
Puerto Rico	6.40	5.03	1.37	1.055	1.029	0.985
Paraguay	6.91	5.53	1.38	1.078	1.057	0.988
Taiwan	6.98	3.49	3.49	1.059	0.945	0.896
Argentina	7.68	6.12	1.56	1.038	1.214	1.149
Poland	8.63	7.65	0.98	1.066	1.150	1.097
Panama	9.14	5.65	3.49	1.019	0.931	0.931
Costa Rica	9.33	6.89	2.44	1.060	0.893	0.854
Venezuela	10.03	6.73	3.30	1.045	0.956	0.924
Un. So. Africa: Asiatics	10.13	6.76	3.37	1.018	0.853	0.844
British Honduras	10.41	8.30	2.11	0.977	1.049	1.072
Nicaragua	10.48	6.93	3.55	1.052	1.114	1.076
Honduras	11.27	5.94	5.33	1.065	1.037	0.954
Portugal	11.28	8.40	2.88	1.055	1.078	1.038
Jordan	12.86	6.93	5.93	1.077	0.861	0.792
Yugoslavia	13.82	10.15	3.67	1.063	0.892	0.845
Chile	14.61	12.60	3.01	1.028	0.966	0.956
Algeria: Moslems	15.26	9.16	6.10	1.090	1.135	1.056
El Salvador	15.54	8.87	6.67	1.042	1.061	1.038
Colombia	15.59	10.00	5.59	1.032	0.991	0.976
México	17.43	9.49	7.94	1.060	0.979	0.933
Ecuador	17.57	10.60	6.97	1.030	0.994	0.986
Bolivia	17.71	9.83	7.88	1.053	0.950	0.887
Peru	18.65	10.81	7.84	1.086	0.993	0.921
Un. So. Africa: Colored	18.85	12.67	6.18	1.019	1.036	1.034
Philippines	19.39	11.30	8.09	1.220	1.118	0.927
Guatemala	22.93	10.39	12.54	1.051	0.980	0.953
Egypt	29.24	14.58	14.66	1.097	1.007	0.915

can be systematically less than unity, implying a preferential mortality of females during this period. We then consider the geographical distribution of the effect and add certain speculations as to its possible role in human evolution.

We have used the data given in the United Nations' *Demographic Yearbook* for 1959,[5] which is largely devoted to natality statistics, in compiling Table 1. This

was prepared in the following way. The mortality under five years of age was first compared with the natality for the year under consideration (in most countries, 1957). It would have been better if a cohort could have been followed through the first five years of life, but the data for this procedure were not always available. The mortality data for the years one to four are likely to be a little low as they refer to a population of neonates that is slightly smaller than that used in the estimate of the mortality in the years less than one, taken as the birth year. The error introduced by our procedure is negligible for our purposes. The ordering of the populations in the table is given by increasing mortality rate under five years.

In columns (2) and (3) of Table 1, the mortality rates computed for the first year and the second to fifth year are given separately. In column (4) we give the neonate sex ratio. Column (5) gives the ratio of boys to girls dying in the population of children in their second to fifth year. Column (6), using the estimate of the proportions of the sexes in the populations as it enters its second year, derived from natality and mortality in the first year, gives a normalized ratio on the assumption of an equal number of boys and girls entering this age category. These figures are subject to the same type of error as are the over-all mortality rates, but in this case the effect is negligible, probably not more than one unit in the decimal place.

Results.—If there were no differential mortality in the second to fifth year, the figures in column (6) should, apart from statistical fluctuation, be unity. Actually there is a very clear and systematic variation. In all the more industrialized and medically advanced populations with low infant (1) and early childhood (2) mortalities, the normalized ratios of column (6) are in excess of unity. This is presumably due to the death of a small number of excess boys who under less good conditions would have died during the first year, partly as the result of genetic and prenatal events, but who are able to survive under very good conditions a little longer.

In most, but not all, of the populations with a mortality rate, below five years, of more than five per cent, the normalized ratio is less than unity; in a number of cases the effect is very striking. In these cases there can be no doubt that the probability of death for a girl is greater than that of a boy during the second to fifth year. In almost every case, an inspection of the crude statistics, which in general cannot be treated by our procedure beyond the fifth year, suggests that this probability is reversed between the fifth and tenth years. The two most dramatic cases are, rather paradoxically, Israel and Jordan, with populations culturally as distant at least superficially, as they are geographically contiguous. Both populations are likely, however, to put disproportionate emphasis on the survival of male children. The few cases of high ratios in populations with high mortalities below five years are probably of considerable interest. The most striking is the South African "colored" population, presumably largely the "Cape Colored" population composed of individuals of partially Hottentot and other aboriginal, and partially of European, descent with some Malayan intermixture. It is most unfortunate that no good data for an indigenous African population south of the Sahara are available.

We can only conclude that the phenomenon of differential mortality of girls in the second to fifth year is widespread though variable and, at least in part, possibly under cultural control.

Detailed Analysis of México.— In view of our original interest in the problem in Central America, it appeared worth while to submit the very extensive data[6]

for México to an analysis by states. These data (Table 2) are probably the best for any country with a total mortality under 5 years of over 15 per cent. Inspection of the data fails to reveal any geographical trend that could be correlated with ethnic, historic, or archaeological findings. The normalized ratios appear more uniform than the unnormalized; in particular, when corrections are made for the very peculiar great excess of males in the infant population of Tabasco, the death ratio falls from an excessive value of 1.075 to a value of 0.903—well within the range of variation for other states.

Evolutionary Significance.—No case has appeared where a large population shows the extraordinary effect observed in the Indian families of the Petén. In the large populations considered in the present paper, there is no reason to believe that the observed differential mortality has any very great effect on the proportion of sexes of reproductive age. Nevertheless, any kind of differential mortality can have selective effects. In the present case any type of behavior on the part of female children

TABLE 2

States of Mexico	Neonate sex ratio (1)	Uncorrected mortality ratio 1–4 (2)	Normalized mortality ratio 1–4 (3)	States of Mexico	Neonate sex ratio (1)	Uncorrected mortality ratio 1–4 (2)	Normalized mortality ratio 1–4 (3)
Baja, California	1.058	1.051	1.027	Guanajuato	1.052	0.899	0.870
Baja, California Sur	1.058	0.891	0.825	Hidalgo	1.082	0.955	0.886
Nayarit	1.068	1.009	0.958	Jalisco	1.054	0.998	0.963
Sinaloa	1.056	1.055	1.003	México	1.054	0.964	0.937
Sonora	1.056	0.972	0.924	Michoacán	1.046	0.991	0.956
Coahuila	1.060	1.010	0.966	Morelos	1.060	0.958	0.909
Chihuahua	1.054	1.027	0.993	Puebla	1.054	0.987	0.957
Durango	1.076	0.998	0.955	Querétaro	1.070	0.931	0.871
Nuevo León	1.050	1.019	0.975	Tlaxcala	1.050	0.885	0.869
San Luis Potosí	1.078	0.994	0.938	Campeche	1.032	1.112	1.064
Tamaulipas	1.064	1.010	0.960	Quintana Roo	1.078	0.963	0.880
Zacatecas	1.048	0.970	0.924	Tabasco	1.180	1.075	0.903
Aguascalientes	1.052	0.993	0.949	Veracruz	1.108	0.985	0.881
Distrito Federal	1.046	0.940	0.923	Yucatán	1.066	0.960	0.923
				Colima	1.080	1.085	1.055
				Chiapas	1.111	1.012	0.920
				Guerrero	1.074	1.039	0.966
				Oaxaca	1.052	1.030	0.990

that reduced their probability of death would, in so far as it had a genetic component, contribute to human evolution.

We have already shown[3] that, under certain circumstances, the differential mortality might be demographically catastrophic. Since in a mild form the effect is very widespread, it is not unlikely that processes preventing its development to an extreme degree are of some importance. We would therefore tentatively suggest that the existence of a mild degree of sexually provocative behavior, which is normally directed by small girls to their fathers and other adult males in contemporary societies, may function as a means of eliciting interest on behalf of such children in a cultural system that in many other ways is directed in favor of males. The genetic basis of variation in such behavior would presumably be of a very general nature, not necessarily sex-specific, involving rather simple variations in relative rates of maturation (cf. Hutchinson[7]), and would not be inconsistent with all the details of the behavior being culturally learned. At least the hypothesis provides a formal explanation of infantile sexuality in man as an adaptive function. At the

same time any evolutionary change accentuating this type of behavior is likely to alter the basis on which adult psychological structure develops.

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[1] Cowgill, U. M., *Trans. Conn. Acad. Arts and Sci.*, **42**, 1 (1961).

[2] Cowgill, U. M., *Amer. Anthropol.*, **64**, 273 (1962).

[3] Cowgill, U. M., and G. E. Hutchinson, *Human Biol.*, **35**, 1 (1963).

[4] The United Nations data (1959) give an extraordinary ratio, consistent over a series of years, below unity for Iran. The figures are large and the results cannot be due to random fluctuations; we suspect a misunderstanding in reporting the data. British Honduras gives ratios below unity in some years but the figures are very small.

[5] United Nations, *Demographic Yearbook* (1959).

[6] México, *Dirección General de Estadística, Anuario Estadístico de Los E.U.M.* (1953).

[7] Hutchinson, G. E., *Amer. Nat.*, **93**, 81 (1959).

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*DIRECT AND OPTICAL ASSAY OF LEAF MASS OF THE LOWER MONTANE RAIN FOREST OF PUERTO RICO**

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Comparative studies of structure and metabolism of large and ancient ecosystems such as the rain forest with a long evolutionary history of competitive survival may show the ability of complex and completely self-maintaining systems to use solar energy. Measurements made of chlorophyll, photosynthesis, and respiration per unit of leaf area can be multiplied by data on leaf area where available to obtain the parameters of structure and function of representative prisms of the ecosystem. For the rain forest, data for this purpose have not been forthcoming and simple methods for such assay are needed.

Means for measuring the total leaf density would also allow monitoring of the effects of radiation sources in forests. This communication reports the use of paired solar cells to monitor the optical density in a plot of the lower montane forest of Puerto Rico plus data on weights and areas of leaves in space prisms sampled within the plot for calibration.

Working in grassy and shrubby communities with selenium cells, Monsi and Saeki[6] have already provided theoretical basis and justification for relating leaf structure and optical extinction.

Study Area.—Studies were conducted in a research tract of the Luquillo National Forest in the El Verde area at 510 m elevation just below the access road at Marker K-4, H-4 in an area described as the Tabanuco type by Wadsworth.[1] In the area are numerous large trees 0.5–1 meter in diameter with tree heights ranging from the general forest top at 18 m to occasional emergent trees on the ridges up to 30 m. Topography is rugged generally with 50 degree slopes or greater, ridges alternating with deep ravines. Rainfall is 238 cm per year with greater than 10 cm every month.

Solar Cells.—As indicated in Figure 1, one solar cell (silicon cell, $1^1/_8$ by $1^1/_8$ in.

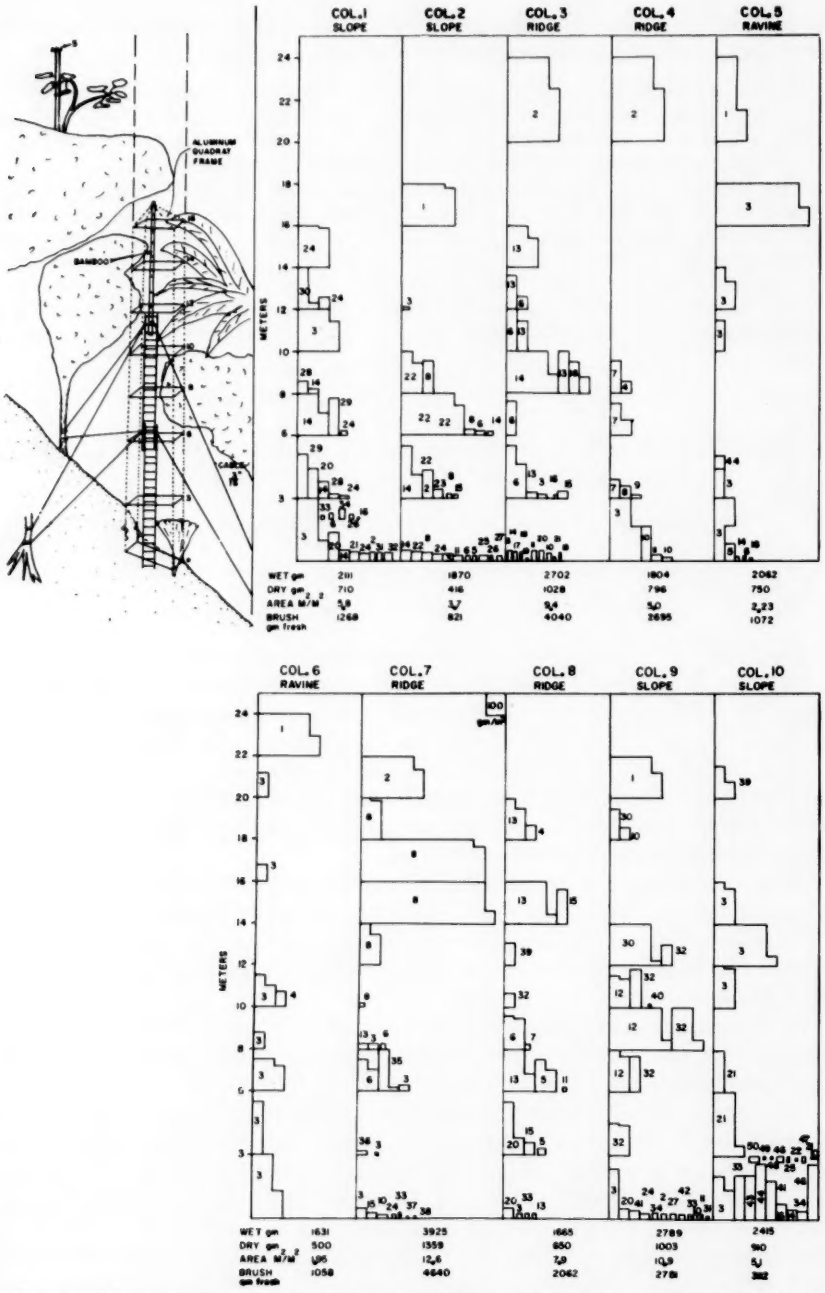

FIG. 1.—Diagram of the vertical aluminum and chain prism apparatus erected within the montane forest with data on the fresh leaf weights by species from the prism collections in ten columns. At the base are also given the totals per square meter of leaf dry weight, leaf area index,

dimensions, SIM of International Rectifier Corporation, El Segundo, California) was waterproofed by enclosure in plastic and paraffin and clamped to a *Cecropia* tree so that the cell was emergent above the forest. A second cell selected for matching responses to the first was placed in various positions on the forest floor. The responses of both cell circuits were graphed together on semilog paper under the same daylight conditions with a series of glass plates as approximate neutral density filters. The resulting graphs were parallel and nearly straight, overlapping so that readings on the microammeter circuit of one cell could be converted to readings on the milliammeter circuit of the other. Thus, optical densities between the top and bottom cells were computed as the $\log_{10}$ (top)/(ground). The forest limbs and leaves in diminishing the light by absorption, diffusion, and reflection permit many direct rays to reach the forest floor in sun flecks and in reflected and scattered rays. Since much of the light energy reaching the forest floor has not passed through leaves above, it has a similar spectral composition to that reaching the companion cell on top of the forest as already pointed out by Richards.[2]

The solar cell has most of its sensitivity of response between 500 and 1000 millimicrons peaking at 800, and therefore is not as sensitive in the green as a selenium cell and is less affected by selective penetration of green light. From 700 to 1,000 millimicrons, including most of the energy, the leaves are 40 per cent or more reflective—serving to deflect and scatter these light components.[3] With weights of petioles and small limbs as great as the leaves per area, considerable light is also extinguished by absorption and reflection from opaque structures.

A 50-junction Eppley pyrheliometer and the solar cell were exposed together on the vertical ladder at column position 8 (Fig. 1) at 1400, October 16, 1962. Both instruments in general light (not sun flecks) showed approximately a straight line on semilog plot of intensity with forest depth, suggesting that solar cells may be used for optical density computations with approximate linearity in this situation. The pyrheliometer extinction coefficient was 0.115/m and the solar cell extinction coefficient was 0.106/m.

Leaf Prisms.—To calibrate the optical density measurements in terms of the leaf density, leaf area, and chlorophyll "A," a volumetric prism was erected in 10 locations in the tract. An aluminum extension ladder was erected almost vertically and guyed by three $^3/_{16}$ in. wire cables, attached as indicated (Fig. 1). Through a pulley on the top of a bamboo pole bolted to the ladder were hoisted square quadrate frames made of $^1/_2$ in. aluminum tubing with an area of 3.76 m^2, suspended by light

and fresh weight of twigs and brush of diameter less than 2.5 cm. The position of the silicon solar cell is indicated on top of the forest. The numbers in the diagrams refer to the species listed as follows: 1, *Cecropia peltata;* 2, *Dacryodes excelsa;* 3, *Euterpe globosa;* 4, *Alchornea latifolia;* 5, *Sloanea berteriana;* 6, *Cordia borinquensis;* 7, *Miconia sp.;* 8, *Chrysophyllum argenteum;* 9, *Oxandra lanceolata;* 10, *Eugenia Stahlii;* 11, *Nectandra sp.;* 12, *Micropholis garciniaefolia;* 13, *Ormosia Krugii;* 14, *Philodendron sp.;* 15, *Nectandra membranacea;* 16, *Marcgravia sp.;* 17, *Meliosma Herberti;* 18, *Lynociera domingensis;* 19, *Comocladia glabra;* 20, *Drypetes glauca;* 21, *Guarea ramiflora;* 22, *Miconia prasina:* 23, *Croton poecilanthus;* 24, *Inga vera;* 25, *Guarea ramiflora;* 26, *Duggenia hersuta;* 27, *Ichnanthes palens;* 28, *Psychotria brachiata;* 29, *Mikania pachyphylla;* 30, *Alchorneopsis portoricensis;* 31, *Athyrocarpus persicariaefolius;* 32, *Ocotea leucoxylon;* 33, unidentified fern; 34, *Piper sp.;* 35, *Meliosma Herberti;* 36, *Nectandra antillana;* 37, *Myrica splendens;* 38, *Neorudolphia volobulus;* 39, *Didymopanax morototoni;* 40, Bromeliad; 41, *Pilea leptofolia;* 42, *Polypodium sp.;* 43, moss; 44, *Peperomia robustior;* 45, broken fragments of species listed in column 10; 46, *Begonia decandra;* 47, *Anthurium sp.;* 48, *Polytaeniomi faeci;* 49, unidentified; 50, unidentified.

chains so as to form a series of space boxes at 2 meter intervals, the lowermost two prisms 3 meters apart. Then one man, operating a pruning saw within the prism, trimmed the leaves to 20 m, dropping limbs and leaves for sorting. For each species representing a significant biomass (100 gm or more) ten leaves were taken for determination of area with planimeter and determination of dry weight after 24 to 48 hr at 105°C. The area-to-wet weight and the dry-to-wet weight conversion factors were used to compute leaf areas and dry weights in the prism.

Two species of trees had emergent branches above 20 m in some quadrats. Giant leaves of *Cecropia peltata* were counted from below by visual projection of the prism. Emergent portions of the dominant Tabanuco (*Dacryodes excelsa*) were estimated from a graph of leaf weight versus tree diameter based on 13 large trees.[4] The trunk diameter of the emergent trees with canopies over the prism provided estimates of leaf weight of the whole crown, which were prorated according to the crown area. A representative limb of sun leaves of this species was cut to obtain 1.72 as the ratio of leaf-to-brush weight of limbs less than 2.5 cm. This conversion was used to estimate limb weight in the emergent portions of columns 3, 4, and 7 (Fig. 1). Column studies were done in pairs 7 m apart (1 and 2, 3 and 4, 5 and 6, etc.) with 14 m from one pair to the next.

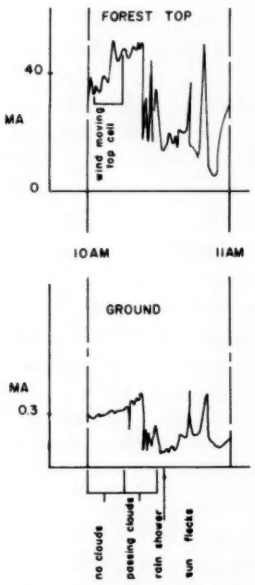

FIG. 2.—Recorder traces of top and ground solar cells on a dual channel, October 19, 1962.

Results.—The type of light trace obtained with the recorder (see Fig. 2) shows the matching responses of the two cells used for computing the apparent optical density. At 0800, October 13, the mean apparent optical density in a ravine at a somewhat sheltered angle was 1.76. On top of the ridge it was 1.22; behind the ridge it was 1.90; and with the bottom cell located within sun fleck spots instead of within the general light field, a mean value was 1.15. The relatively high and variable optical densities reflect the varying paths of light entering the sharply changing angles of the rugged forest topography in the morning with the sun's rays at a low angle and transverse to the alternating ravine and ridge structure.

At noon on October 15, a mean apparent optical density was more uniform, 1.37 in the ravine and 1.38 on the ridge. The sun at this time was 13 degrees south of the vertical and entering the steep northward-facing slope at approximately 59 degrees from the normal to the forest plant, parallel to the ravine and ridge structure, thus minimizing local shadowing found in the morning data.

In the afternoon at 1600 with sun falling almost perpendicular to the westward-facing slope, where the afternoon sun supports more than usual herbaceous vegetation (Fig. 1, col. 10), the mean apparent optical density observed was 0.90, the minimum value obtained.

In Figure 1 are the data on the distribution of the leaves in the 10 prisms with totals at the base. The mean leaf area index referred to the vertical was 6.4 m^2/m^2

ranging from 1.95 in the palm-filled ravines to 12.6 on top of the Tabanuco-covered ridge. This value is similar to the 7.3 previously computed with assumptions about leaves in a plot at Sabana.[4] Ogawa, Yoda, and Kira,[5] computing data of Aubreville and Jones and using some data conversions from Thailand, report 8.5 for tropical rain forest of the Ivory Coast and 9.5 from Nigeria.

If the value of 6.4 m^2 of leaves per m^2 of horizontal plane is projected to the forest normal by using a cosine of 50 degrees for the land slope, a value of 4.5 results corresponding to the optical density of 0.90. If projected to an angle with the vertical of 13 degrees to correspond to the angle of the sun's incidence at noon, a leaf area index of 7.5 corresponds to the apparent optical density at noon of 1.37. Calibration of optical densities were thus made with trigonometric projection of leaf prism data along the light path.

Following Monsi and Saeki[6] the extinction coefficient per unit of leaf area (K) is related to the leaf area index (F), the incident light (I_0) and the interior light (I) with the relation $I = I_0 \exp -KF$. For the Tabanuco forest this extinction coefficient K is 0.18, close to that predicted by Monsi and Saeki[6] as optimal for a community adapted to high light intensities, where the component leaves possess a Michaelis hyperbolic relationship of photosynthesis per leaf to light intensity.

The mean coefficient of variation (standard deviation/mean) for 5 groups of replications on the ridge was 12.3 per cent (12.9, 11.9, 12.9, 9.7, 14.1); for 4 groups of replications in the ravines and behind ridges where optical paths were longer, the mean value was 7.2 per cent (12.4, 5.5, 8.6, 2.4). These values indicate more homogeneity in optical densities from light data compared to prism determinations with a coefficient of variation of 97.6 per cent.

Using optical density as a measure of the leaf and limb biomass just as in colorimetry, a rapid means is thus provided for appraisal of the state of forests regarding their principal producing tissues. In the Tabanuco type of the lower montane rain forest of Puerto Rico, an optical density of 1.2 is equivalent to a leaf fresh weight of 2297 gm/m^2 (including supporting limb structure), a dry weight of 812 gm/m^2, and a leaf area of about 6.4 m^2/m^2. Since other kinds of forests have leaves of other textures, prism calibrations will be required of each.

As an example of the use of leaf data, one may estimate the chlorophyll "A" for this area of the lower montane rain forest by multiplying the leaf area index of 6.4 by the mean chlorophyll "A" per area of leaf of 0.34 gm/m^2 for 19 species (from an unpublished manuscript by Odum, Abbott, Selander, Golley, and Wilson[7]) to obtain 2.2 gm/m^2.

Using a net photosynthetic rate of 0.10 gm C/m^2 of leaf area typical of some measurements within the crown and middle story of this forest,[7] one may compute a forest photosynthetic rate of 0.64 gm C/m^2/hr. Using half of 400 kilocalories per hr insolation per square meter in the middle of the day as photosynthetically available and a conversion of 8.6 K-Calories per gram, one may compute about 3 per cent efficiency of net photosynthesis.

These chlorophyll and efficiency values are not unlike those of other natural and engineered photosynthetic systems operating on sunlight. If, in the rain forest's long evolutionary history, natural selection has already worked toward maximum yield, the ordinary values found in this ancient ecosystem may imply inherent limits of a thermodynamic nature.

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RADIATION RECOVERY ENHANCED THROUGH INHIBITORS OF PROTEIN SYNTHESIS AND AMINO ACIDS

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Over a period of several years we have sought to develop a procedure where initial damage from radiation and recovery from this damage could be examined individually. The production of two-hit chromosomal aberrations by X rays in the microspores of *Tradescantia paludosa* in a helium gas atmosphere has provided a means for this type of investigation.

With a total dose of 400 r at 30°C, we found that in helium there was no time-intensity factor. That is, irradiation at 1 r per min gave the same aberration yield (0.66 per cell) as a pretreatment in helium for equal period of time, 400 min, followed by irradiation at 50 r[6] or at 400 r (unpublished) per min. Also, with no pretreatment in helium but with irradiation, both at 50 r[6] and 400 r[7] per min, the aberration yields were the same. Furthermore, the data from fractionation experiments, where the total dose of 400 r was given in two equal amounts, showed that the second fraction could be given from 0–1½ hours after the first without modifying the aberration yield.[3] With a longer period of time between the two fractions, the aberration yield actually increased.[3] All these data lead us to the conclusion that the breaks which are capable of becoming involved in forming two-hit aberrations remain open during experimentation in helium.

Additional experimental data which add to the usefulness of the above indicate

that when excess energy is made available within the cell, the aberration yield can be decreased to 0.09 per cell[5], and when energy is limited within the cell, an increase to 0.66 per cell[3, 6] can be obtained. The utilization of these approaches in studying radiation damage provides a rather sensitive method for measuring the influence of various chemical compounds on the recovery process. The initial problem under investigation was the effect of inhibitors of protein synthesis on chromosome repair, but subsequent data necessitated also studying the influence of some of the amino acids on chromosome repair.

Dihydrostreptomycin, puromycin, and chloramphenicol, which were used in these experiments, have been reported by several investigators to inhibit protein synthesis by preventing the amino acid transfer from S-RNA to protein. Among such reports for dihydrostreptomycin are those of Erdös and Ullmann,[10] Stachiewicz and Quastel,[18] and Eaton and Caffrey;[9] for puromycin, Yarmolinsky and De La Haba,[20] Nathans and Lipmann,[16] and Gorski, Aizawa, and Mueller;[13] and for chloramphenicol, Brock[8] (a review of 102 papers), and Yee, Pan, and Gezon.[21] While protein synthesis is inhibited by these antibiotics, a number of other processes in the cell are only slightly or not at all affected. Some of these are the synthesis of RNA, DNA, nucleotides, phospholipids, polysaccharides, cell walls, and simple peptides such as glutathione. The antibiotics do not inhibit oxidative, hydrolytic, or degradative processes, the uptake of metabolites into the cell, or the rate of ATP generation within the cell.

Materials and Methods.—Inflorescences of *Tradescantia paludosa* were removed just prior to use from plants growing in the greenhouse and after treatment returned to the greenhouse in beakers of water which were placed in a constant-temperature chamber of 30°C. Acetocarmine preparations were made of the microspores 96 hr after irradiation and chromosomal aberrations of the ring and dicentric types were scored.

All X-radiations were given at 30°C and at 50 r per min for 8 min. All irradiated material was posttreated for 15 min in the gas used during irradiation.

The inflorescences to be treated with one of the chemicals were cut to $2^1/_2$ in. in length and placed in a 50 ml beaker, into which 50 ml of the solution was poured. All experimental material was preirradiatively treated in the solution in air for $1^1/_2$ hr.

The dihydrostreptomycin sulfate, puromycin dihydrochloride, and the amino acids were obtained from the Nutritional Biochemicals Corporation, while the chloramphenicol was obtained from Parke, Davis and Co. from Lot No. 185212 A, which was made up for investigational purposes. The amount of chemical used in each experiment is given with the experimental data.

Results and Discussion.—In order to determine whether any of the three antibiotics had any radiomimetic properties, inflorescences were placed for $1^1/_2$ hr in the most concentrated solution for each chemical used. Acetocarmine preparations were made 96 hr later. In each of the three experiments, 500 metaphase configurations were examined for aberrations of the ring and dicentric types. No such aberrations were found.

Three control experiments were carried out for the data reported here on antibiotics. In each of two experiments 500 metaphase configurations were observed, while in one only 300 were counted. The combined results indicate that the chro-

TABLE 1

EFFECT OF PREIRRADIATION TREATMENT IN ANTIBIOTICS ON ABERRATION YIELD IN *Tradescantia* MICROSPORES

Treatment	mg/100 ml of water	No. cells	Av. interchanges per cell
Control		1300	0.24 ± 0.01
Dihydrostreptomycin	146	500	0.21 ± 0.02
"	72	500	0.19 ± 0.02
"	36	500	0.14 ± 0.02
"	7.5	500	0.16 ± 0.02
Puromycin	110	500	0.17 ± 0.02
"	55	500	0.21 ± 0.02
"	27	500	0.22 ± 0.02
"	6	500	0.21 ± 0.02
Chloramphenicol	90	500	0.17 ± 0.02
"	60	500	0.18 ± 0.02
"	30	450	0.17 ± 0.02
"	6.5	500	0.16 ± 0.02

mosome aberration yield was 0.24 ± 0.01 per cell (Table 1). Any chemical treatment of the material which in any manner prevented rejoining would be expressed in an increase in aberration yield, while any treatment which facilitated rejoining in any manner would result in a reduction in aberration yield. (The extremes so far obtained with other methods under the same experimental conditions are a low of 0.09 aberrations per cell and a high of 0.66.)

Four different solutions of dihydrostreptomycin were used, representing a range in concentrations from 7.5 mg/100 ml of water to 146 mg/100 ml. In all four experiments the aberration yields (Table 1) were less than the control value of 0.24. The two lower concentrations of the antibiotic used, 36 and 7.5 mg produced the greatest effect by reducing the aberration yield to 0.14 and 0.16 per cell.

The concentrations of the four solutions of puromycin used ranged from 6m g/100 ml of water to 110 mg, all of which resulted in aberration yields lower than the control. With this antibiotic the highest concentration used, 110 mg/100 ml, gave the lowest aberration yield, which was 0.17 per cell.

The four different concentrations of chloramphenicol used, 90, 60, 30, and 6.5 mg/100 ml of water, all produced about the same reduction in aberration yield, around 0.17 per cell.

In general the expression of all three inhibitors of protein synthesis was a reduction in aberration yield from that of the untreated control irradiated in helium. The reduction in aberration yield is interpreted as indicating that, in this material, not only is protein synthesis not necessary for chromosome rejoining, but related to the action of the drugs, some additional energy is made available for chromosome rejoining, thereby bringing about a decrease in chromosomal aberrations.

Since the action of these antibiotics is rather specific, in that they prevent the amino acid transfer to protein, the additional energy realized from their use could come both from the absence of protein synthesis and from the accumulation of amino acids. In relation to the first suggestion, Yarmolinsky and De La Haba[20] and Hoagland[15] found that ATP is used in the amino acid transfer from S-RNA to protein. Thus, inhibition of protein synthesis could make more ATP available within the cell. Beatty and Beatty[4, 5] have shown in *Tradescantia* that the use of exogenous ATP, under the same experimental conditions used here, reduces the aberration yield from 0.24 of the control to 0.14 per cell. In relation to the second suggestion, Hancock[14] found a rapid increase in the size of the free amino acid pool

after addition of chloramphenicol to the cultures and that this rate of accumulation in the pool becomes quite close to the rate at which these amino acids had been incorporated into proteins. Estimations were made of 12 major components which constituted approximately 70% of the total pool in normal cells. These were glutamic acid, asparatic acid, proline, histidine, glycine, alanine, lysine, valine/methionine, and leucine/isoleucine/phenylalanine. The minor components which were present in very small amounts were serine, threonine, cysteine, tyrosine, tryptophan, and arginine.

Experiments were carried out with *Tradescantia* using these eighteen amino acids in addition to ornithine, cystine, citrulline, and hydroxy proline. In all cases the inflorescences to be used were treated in air for $1^1/_2$ hr in solutions at the concentrations listed in Table 2 and irradiated in helium. The results in Table 2 indicate

TABLE 2

INFLUENCE ON CHROMOSOMAL ABERRATION YIELD OF $1^1/_2$ HR PRETREATMENT IN AMINO ACID SOLUTIONS FOLLOWED BY IRRADIATION

Treatment	No. cells	Av. interchanges per cell
Helium control	2300	0.24 ± 0.01
L citrulline 3×10^{-3} *M*	500	0.14 ± 0.02
L histidine 3×10^{-3} *M*	500	0.15 ± 0.02
" 6×10^{-3} *M*	500	0.17 ± 0.02
L proline 3×10^{-3} *M*	500	0.16 ± 0.02
" 6×10^{-3} *M*	500	0.19 ± 0.02
L glycine 1.5×10^{-3} *M*	500	0.17 ± 0.02
" 3×10^{-3} *M*	1000	0.17 ± 0.01
" 1×10^{-2} *M*	500	0.17 ± 0.02
DL phenylalanine 1.5×10^{-3} *M*	500	0.17 ± 0.02
L cysteine 1.5×10^{-3} *M*	500	0.18 ± 0.02
L isoleucine 1.5×10^{-3} *M*	500	0.18 ± 0.02
L alanine 3×10^{-3} *M*	500	0.19 ± 0.02
L threonine 3×10^{-3} *M*	500	0.19 ± 0.02
DL serine 3×10^{-3} *M*	500	0.20 ± 0.02
Hydroxy L proline 3×10^{-3} *M*	500	0.21 ± 0.02
L valine 3×10^{-3} *M*	500	0.21 ± 0.02
L methionine 3×10^{-3} *M*	500	0.21 ± 0.02
L tyrosine 1.5×10^{-3} *M*	500	0.22 ± 0.02
DL cystine 1.5×10^{-3} *M*	500	0.23 ± 0.02
DL aspartic acid 3×10^{-3} *M*	500	0.23 ± 0.02
L arginine 3×10^{-3} *M*	400	0.23 ± 0.02
L ornithine 3×10^{-3} *M*	500	0.23 ± 0.02
L tryptophan 3×10^{-3} *M*	500	0.24 ± 0.02
L lysine 3×10^{-3} *M*	200	0.24 ± 0.04
DL leucine 1.5×10^{-3} *M*	500	0.24 ± 0.02
L glutamic acid 1.5×10^{-3} *M*	200	0.24 ± 0.04

that the effect of many of the amino acids is shown by the reduction of the aberration yield. Citrulline, histidine, proline, glycine, phenylalanine, cysteine, isoleucine, alanine, threonine, and serine definitely reduced the aberration yield with the first three listed being most effective. Hydroxy proline, valine, methionine, and perhaps tyrosine had doubtful effects, while cystine, asparatic acid, arginine, ornithine, tryptophan, lysine, leucine, and glutamic acid had little or no effect.

The amino acid effect found in these experiments is believed to have been accomplished through the acquisition of additional energy for chromosome rejoining in the treated material in contrast to the nontreated material. This conclusion is based upon the results from other experiments where postirradiative treatment of material for $1^1/_2$ hr in ATP,[4] potassium gluconate,[4] and glutathione[5] gave the same results as the preirradiative $1^1/_2$ hr treatment, indicating that the effect was on the

recovery process rather than on initial damage or by a protective process during irradiation. Energy from amino acids whether supplied exogenously or endogenously by an increase in the amino acid pool could come from the operation of inhibitory feedback mechanisms and from catabolism of amino acids. Ames and Hartman[2] stated that histidine production in Salmonella is regulated by two control mechanisms, histidine inhibition of the first enzyme and histidine repression of the synthesis of all the histidine biosynthetic enzymes. In the synthesis of histidine ATP is utilized. If in *Tradescantia* the same biosynthetic pathways exist, the ATP gain through inhibition of this pathway through an increase in histidine content in the cell could be used by the chromosomes for rejoining. Similar feedback mechanisms could account for additional energy.

Since proline was one of the three most active amino acids in reducing aberration yield, it will be used to illustrate how additional energy could be derived from catabolism of an amino acid. In the change of proline to pyrolline carboxylate by the enzyme proline dehydrogenase, DPNH is produced. Beatty and Beatty[5] have shown that a concentration of 1.5×10^{-3} *M* of DPNH was able to reduce the aberration yield from 0.24 of the control to 0.11 per cell. Thus, the catabolism of proline as illustrated or of other amino acids could provide additional energy in the cell which could be used for chromosome repair.

Several investigators using other materials have reported increased recovery from radiation damage following the use of these antibiotics. Gillies and Alper[12] found that in *Escherichia coli* B the lethal effects both of X rays and ultraviolet light were greatly reduced if the bacteria were incubated for a period after irradiation on solid medium containing chloramphenicol. Allison *et al.*[1] reported that under their experimental conditions the total number, as well as the number of viable cells, of *Escherichia coli* in liquid BHI culture increased for a considerable period of time after the addition of chloramphenicol. Gale and Folkes[11] observed stimulation of RNA synthesis by chloramphenicol in *Staphylococcus aureus;* Yee, Pan, and Gezon[21] reported similar results in *Shigella flexneri* 3 B-1003; and Rabinovitz and Fisher[17] reported that the labeling of soluble proteins was doubled in Ehrlich ascites cells treated with puromycin. Wolff,[19] however, did not find a decrease in chromosome aberration yield in Vicia. By administering an antibiotic between two doses of radiation, he found that the breaks from the first dose remained open for at least 75 min, in contrast to 30 min for the controls.

The recovery from radiation damage in *Tradescantia* as measured by chromosome aberrations seems to be an energy-related phenomenon which can be manipulated, within limits, by modifying the amount of energy within the cell, either by exogenous or endogenous means. We believe that the reduction in aberration yield in the experiments reported here came about through the release of additional energy within the cell initiated by the treatment of the material with the inhibitors of protein synthesis and with some of the amino acids.

Summary.—*Tradescantia* inflorescences which were preirradiatively treated for $1^1/_2$ hr in protein inhibitors, dihydrostreptomycin, puromycin, or chloramphenicol, and irradiated in helium, gave lower chromosomal aberration yields than did the chemically untreated controls, indicating that, in these experiments, protein synthesis was not necessary for chromosome rejoining and that material treated with these antibiotics showed more recovery from radiation damage than the controls.

The recovery is thought to be due to the presence of additional ATP brought about by the inhibition of protein synthesis and through the addition of amino acids in the pool. The amino acids could provide additional ATP through inhibitory feedback mechanisms and the catabolism of amino acids. The exogenous use of citrulline, histidine, proline, glycine, phenylalanine, cysteine, isoleucine, alanine, threonine, and serine reduced the aberration yield to a value approximating that obtained with the antibiotics.

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DECREASE OF POPULATION FITNESS UPON INBREEDING

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The discussion in this communication is confined to two types of stable genetic equilibria with respect to a single locus. One is that due to higher reproduction of the heterozygote (*Aa*) over the homozygotes (*AA* and *aa*), and will be referred to as the *heterotic* equilibrium for brevity. Another is that selection balanced by recurrent mutations and will be referred to as the *mutational* equilibrium. Both types are known to exist in natural random mating populations and examples of each type may also be found in human populations.

Inbreeding, as exemplified by consanguineous marriages in man, increases the frequency of homozygotes in the population and decreases the frequency of heter-

ozygotes. In recent years it has been claimed[1] that mortality and morbidity data on inbred offspring from consanguineous marriages enable us to decide whether deleterious genes (causing disease or death) have been maintained by heterotic or by mutational equilibrium, because, it is said, these two types of genes have differential response to inbreeding. This claim has stimulated a number of inbreeding studies and review of old ones. Inference has been drawn that the deleterious loci must be in mutational equilibrium. The conclusion of the present analysis, however, directly contradicts the current belief.

Heterotic Equilibrium.—Let w_1, w_2, w_3 be three numbers to denote the relative "fitness" (reproductive ability) of the three genotypes AA, Aa, aa, respectively. It is the ratio $w_1:w_2:w_3$ that specifies the selection force. The case $w_1 < w_2 > w_3$ leads to heterotic equilibrium. In Table 1 are shown three arbitrary systems of notation for the w's and

$$\begin{array}{rccc} & w_1 & : w_2 & : w_3 \\ = & 1 & : 1 + Hs & : 1 - s \\ = & 1 - t & : 1 & : 1 - r \\ = & 1 - b & : 1 + \sqrt{bc} & : 1 - c \end{array}$$

so that the three systems of notation are describing the same selection scheme. The letters s, t, r, b, and c are all positive fractions and H is positive. The frequencies of alleles A and a are denoted by p and q, where $p + q = 1$. At equilibrium, the gene frequency after the operation of selection must be equal to that before selection. Hence, the equilibrium condition is, in general,

$$q = \frac{pqw_2 + q^2w_3}{p^2w_1 + 2pqw_2 + q^2w_3}$$

which leads to the solution

$$q = \frac{w_2 - w_1}{(w_2 - w_1) + (w_2 - w_3)}.$$

TABLE 1

EQUILIBRIUM GENE FREQUENCY AND AVERAGE FITNESS OF A RANDOM MATING POPULATION UNDER THREE SYSTEMS OF NOTATION FOR FITNESS (w)

Genotype	Frequency f	System I w	System II w	System III w
AA	p^2	1	$1 - t$	$1 - b$
Aa	$2pq$	$1 + Hs$	1	$1 + \sqrt{bc}$
aa	q^2	$1 - s$	$1 - r$	$1 - c$
Equilibrium	q	$\frac{H}{1 + 2H}$	$\frac{t}{r + t}$	$\frac{\sqrt{b}}{\sqrt{b} + \sqrt{c}}$
Average	$\bar{w}_0$	$1 + Hsq$	$1 - rq$	1.00

The explicit expression for the equilibrium value of q under each system is shown in Table 1. It may readily be verified that

$$q = \frac{H}{1 + 2H} = \frac{t}{r + t} = \frac{\sqrt{b}}{\sqrt{b} + \sqrt{c}}$$

by virtue of the conversion relationships from one system to another:

$$t = 1 - \frac{1}{1 + Hs} = \frac{Hs}{1 + Hs}, \quad r = 1 - \frac{1 - s}{1 + Hs} = \frac{s + Hs}{1 + Hs} \qquad \text{(I–II)}$$

$$t = 1 - \frac{1 - b}{1 + \sqrt{bc}} = \frac{b + \sqrt{bc}}{1 + \sqrt{bc}}, \quad r = 1 - \frac{1 - c}{1 + \sqrt{bc}} = \frac{c + \sqrt{bc}}{1 + \sqrt{bc}} \qquad \text{(II–III)}$$

$$Hs = \frac{b + \sqrt{bc}}{1 - b}, \quad s = \frac{c - b}{1 - b}, \quad H = \frac{b + \sqrt{bc}}{c - b} \qquad \text{(I–III)}$$

Since $w_1 > w_3$ in our example, we have $c > b$, so that $c - b$ is positive in the expressions above.

The equilibrium value of q is thus *invariant* with regard to arbitrary systems of notation. It is evident that this is the case from the general expressions. It is due to this invariant property of equilibrium that geneticists usually pay little attention to systems of notation.

The Average Fitness.—When we come to quantities that are not invariant with regard to notation systems, each arbitrary system will exhibit its own peculiar properties (artefacts, if you wish) which require special interpretation from a particular point of view. For instance, the so-called average fitness of the population

$$\bar{w}_0 = \sum f w = p^2 w_1 + 2pqw_2 + q^2 w_3$$

is such a quantity. The subscript 0 indicates random mating. The quantity $\bar{w}_0$ varies from system to system and does not have a constant meaning by itself. Its value at equilibrium condition is given in the bottom row of Table 1, showing that under system I, it is always greater than unity; under system II, it is always smaller than unity; and under system III, it is exactly unity. And yet it refers to the same population, subject to the same selection scheme, and with the same equilibrium condition. Evidently, $\bar{w}_0$ does not represent any inherent property of a population at all. The value of $\bar{w}_0$ of one population obtained from one system of notation cannot be directly compared with the $\bar{w}_0$ of another population obtained from a different system of notation.

The particular meaning of $\bar{w}_0$ in our example is as follows. *System I:* If the allele a is absent from the population, all individuals (AA) would have the same fitness and there is no genetic selection. We describe this situation by saying that the population has an average fitness of unity. Then $\bar{w}_0 = 1 + Hsq$ means that the population as a whole is better off with allele a than without it, because of the higher reproductive ability of the heterozygotes. *System II:* The highest fitness value, of whatever genotype it happens to be, is taken as the "standard." In this case, the fitness of Aa is the standard with which the other genotypes are compared. Then $\bar{w}_0 = 1 - rq$ means the population is worse off than the *hypothetical* (nonexistent in nature under panmixia) population consisting of nothing but heterozygotes. The expression $\bar{w}_0 = 1 - rq$ is merely another way of saying that the heterozygote has the highest fitness value. *System III:* Since equilibrium implies that the population remains the same from generation to generation, the selection scheme neither confers any gain nor inflicts any loss on the population. Then $\bar{w}_0 = 1$ simply means that the genetic composition of the population stays the same way under the prevailing condition. It is important to note that there

is no contradiction among the three "interpretations," each being a statement from a particular viewpoint. On the other hand, it is clear that no fixed and intrinsic meaning can be attached to $\bar{w}_0$.

From the general expression for q, we see that widely different selection schemes may lead to identical equilibrium conditions. Other difficulties in interpreting values of $\bar{w}_0$ have been discussed by the author[2] previously.

Decrease in Average Fitness on Inbreeding.—In order to amplify the effect of inbreeding on the average fitness, we render the existing equilibrium population to complete homozygosis without changing its gene frequency. This is equivalent to making each allele (A and a) in the existing population double itself, so that there will be p AA and q aa in the complete homozygous state, where the p (or q) is the same as that in the original random mating equilibrium population. The values of the new average fitness, $\bar{w}_1$, are given in Table 2, where the subscript 1 denotes the state of complete homozygosis. Comparing the $\bar{w}_0$ of Table 1 with $\bar{w}_1$ of Table 2, we see that the latter is smaller than the former under all systems of notation. It is a biological fact that inbreeding decreases the average fitness of a heterotic population. The problem is how to assess the decrease. What sort of an index should we use to measure the extent of the decrease?

TABLE 2

AVERAGE FITNESS OF A COMPLETELY HOMOZYGOUS POPULATION WITH THE SAME GENE FREQUENCY AS IN TABLE 1

Genotype	Frequency f	System I w	System II w	System III w
AA	p	1	$1 - t$	$1 - b$
aa	q	$1 - s$	$1 - r$	$1 - c$
Average	$\bar{w}_1$	$1 - sq$	$1 - 2rq$	$1 - \sqrt{bc}$

Using notation system II, Crow (1958) defines the quantity $L = 1 - \bar{w}$ as the "genetic load" of a population. Thus, at the random mating state, the load is $L_0 = 1 - \bar{w}_0 = rq = rt/(r + t)$; and at the homozygous state, the load is $L_1 = 1 - \bar{w}_1 = 2rq = 2rt/(r + t)$. From this observation he concludes that the doubling of genetic load ($L_1/L_0 = 2$) by inbreeding is characteristic of a heterotic equilibrium population, apparently without realizing that this is only a consequence of the particular notation system he has adopted, and not an inherent property of the population at all. This is made amply clear by the accompanying systems I and III which, to be sure, are describing exactly the same event. Under system I, the definition of load gives $L_0 = -Hsq$, and $L_1 = sq$, so that $L_1/L_0 = -1/H$ which may be very large or very small numerically but always negative. Under system III, $L_0 = 0$ and the ratio L_1/L_0 is "infinity." This shows that the concept of genetic load, as defined by $1 - \bar{w}$, is not usable to measure the extent of decrease in average fitness, because $\bar{w}$ itself varies from system to system.

Any true measurement of the decrease in average fitness must be an *invariant* with regard to the arbitrary notation systems. The simplest index for this purpose is the ratio $\bar{w}_1/\bar{w}_0$ which remains the same, whatever the notation system. That is,

$$\frac{\bar{w}_1}{\bar{w}_0} = \frac{1 - sq}{1 + Hsq} = \frac{1 - 2rq}{1 - rq} = 1 - \sqrt{bc},$$

as may be readily verified, using the conversion relationships (I–II, etc.). Hence,

the ratio $\bar{w}_1/\bar{w}_0$ describes an inherent property of the population. The fraction $1 - \bar{w}_1/\bar{w}_0 = \sqrt{bc}$ measures the percentage decrease of the average fitness for a heterotic equilibrium population when rendered to complete homozygosis.

When the two extreme values, $\bar{w}_0$ and $\bar{w}_1$, are known, the average fitness of a population with any inbreeding coefficient (F) may be readily obtained by the relation

$$\bar{w}_F = (1 - F)\bar{w}_0 + F\bar{w}_1 = \bar{w}_0 - F(\bar{w}_0 - \bar{w}_1).$$

The decrease in average fitness is then measured by the ratio

$$\frac{\bar{w}_F}{\bar{w}_0} = 1 - F\left(\frac{sq + Hsq}{1 + Hsq}\right) = 1 - F\left(\frac{rq}{1 - rq}\right) = 1 - F\sqrt{bc}$$

which is invariant with regard to notation systems. When $F = 1$, it reduces to $\bar{w}_1/\bar{w}_0$. For the special group of offspring from first cousin marriages, the percentage decrease is $^1/_{16}\sqrt{bc}$.

Mutational Equilibrium.—The selection scheme for mutational equilibrium is $w_1 > w_2 > w_3$, so that the decrease in q due to selection in each generation is balanced by new mutations from allele A to allele a. Let the three fitness values be

$$1,\ 1 - hs,\ 1 - s,$$

where h and s are both positive fractions (analogous to system I). The equilibrium condition for a random mating population is

$$p = \left\{\frac{p - pqhs}{1 - 2pqhs - q^2s}\right\}(1 - \mu)$$

which simplifies to

$$(1 - hsq)\mu = sq(q + h - 2hq)$$

where μ is the mutation rate from allele A to allele a per generation. The average fitness at equilibrium is

$$\bar{w}_0 = (1 - hsq)(1 - \mu).$$

Again, $\bar{w}_0$ varies from system to system and has no fixed meaning. Its properties are strictly peculiar to the particular notation system employed. In our example, $h = 0$ implies that the deleterious gene is recessive. The equilibrium condition would then be $\mu = sq^2$ and $\bar{w}_0 = 1 - sq^2 = 1 - \mu$. In the general case where $h \neq 0$, the factor $(1 - \mu)$ of $\bar{w}_0$ may still be regarded as due to selection against homozygote aa and the factor $(1 - hsq)$ as due to the additional selection against heterozygote Aa.

When this population is rendered to complete homozygosis without changing the gene frequency, the average fitness will be $\bar{w}_1 = 1 - sq$. The relative decrease in population fitness is measured by the invariant ratio

$$\text{(mutational)}\ \frac{1 - sq}{(1 - hsq)(1 - \mu)} > \frac{1 - sq}{1 + Hsq}\ \text{(heterotic)}.$$

We see that the ratio $\bar{w}_1/\bar{w}_0$ for a heterotic equilibrium population is always smaller

than the corresponding ratio for a mutational equilibrium population. In other words, the relative decrease in population fitness upon inbreeding is always greater for a heterotic equilibrium population than for a mutational one.

Crow's conclusion is exactly the opposite to ours. The reason is, again, that he uses the "mutational load" rather than an invariant index. When q is small in comparison with h, the equilibrium condition is then $\mu = hsq$ approximately, and $\bar{w}_0 = 1 - 2hsq$. In his terminology, the respective loads are $L_1 = sq$, and $L_0 = 2hsq$, so that $L_1/L_0 = 1/2h$. If h is somewhere between 0.02 and 0.05, the load L_1 would be 10 to 25 times as large as L_0! On the other hand, $L_1 = 2L_0$ under system II for a heterotic equilibrium population. Combining these two artefacts, he reaches the startling conclusion that the population fitness decreases on inbreeding to a much greater extent for a mutational equilibrium population than for a heterotic one! The evidence based on the ratio $\bar{w}_1/\bar{w}_0$ points to the contrary.

Similarly, for a population with inbreeding coefficient F, the "load" $= hs[2pq(1 - F)] + s[q^2(1 - F) + qF]$ cannot be directly used for any comparison purpose.

A very interesting special case is that when $w_2 = {}^1/_2(w_1 + w_3)$; that is, $h = {}^1/_2$ under the particular notation system employed in our example. Then the equilibrium condition becomes $\mu = {}^1/_2sq/(1 - {}^1/_2sq)$, and the average fitness is

$$\bar{w}_0 = (1 - {}^1/_2sq)(1 - \mu) = 1 - sq = \bar{w}_1 = \bar{w}_F.$$

There is no decrease in average fitness at all with any degree of inbreeding. (For a heterotic population, there is necessarily some decrease.) Furthermore, when w_2 is closer to w_3 than to w_1 (that is, $h > {}^1/_2$), there will actually be an *increase* in population fitness!

Discussion.—The foregoing analysis is algebraically exact and involves no approximations. (The approximation $\mu = hsq$ is Crow's.) It is intended to bring out all the details of the problem. However, even without the analytical exhibition, the qualitative result should be obvious to everyone. The two selection schemes considered are as follows:

	AA	Aa	aa
heterotic	w_1	$<$ w_2 $>$	w_3
mutational	w_1	$>$ w_2 $>$	w_3

Inbreeding eliminates Aa from the population. In the heterotic case, it eliminates the individuals with the highest fitness. In the mutational case, it eliminates the individuals with the intermediate fitness. It follows that inbreeding will cause a greater drop in population fitness for a heterotic population than for a mutational one with the same gene frequency.

To study exact algebraic relationships is one thing. To draw useful conclusions from observed data is quite another. Although we have shown that the decrease in population fitness is greater for a heterotic population than for a mutational one, the actual difference is very small indeed, because hsq, μ, and Hsq are very small quantities for rare harmful genes. Hence, our *practical* conclusion is that the two types of genes (with the same frequency) do not respond to inbreeding differently to any appreciable extent. This could be a great disappointment to those who rely on consanguineous data to distinguish the genes of one equilibrium

type from another. Inbreeding studies are essentially studies of rare gene frequencies in the existing population rather than how and why the gene is maintained in the population.

Summary.—The extent of decrease in population fitness on inbreeding is measured by

$$\frac{\bar{w}_1}{\bar{w}_0} = \frac{\text{average fitness at complete homozygosis state}}{\text{average fitness at random mating state}}$$

Populations in heterotic equilibrium show a greater percentage decrease than populations in mutational equilibrium, but the difference is very small for rare genes. Mortality and morbidity data on inbred offspring from consanguineous marriages cannot distinguish genes in one type of equilibrium from those in another. The current concept of genetic load is based on artefacts rather than on inherent properties of populations, and it leads to erroneous conclusions.

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*SOMATIC RECOMBINATION IN PSEUDOWILD-TYPE CULTURES OF NEUROSPORA CRASSA**

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In the original investigations[1, 2] of pseudowild-type strains, or heterokaryons believed to originate as nondisjunctional disomics in crosses of linked mutants, it was hoped that certain of these strains would prove to have some degree of stability and might serve experimentally as a source of intranuclear heterozygosity. It was possible to show, however, that the disomic (n + 1) condition was unstable and the pseudowild-type (PWT) cultures became heterokaryotic during growth.[2] Subsequent studies on the stability of disomic nuclei indicated that in a significant proportion of PWTs haploidization may have occurred by the end of the second postmeiotic division.[3] Analyses of crosses of PWT strains have failed to reveal any evidence of stable disomic nuclei, and the multinucleate condition of the conidia has prevented ruling out the existence of some disomic nuclei in mature cultures because of the problem of distinguishing a conidium which contains a heterozygous nucleus from one which is heterokaryotic. An analysis of a limited number of microconidial (uninucleate) PWT cultures indicated that disomic nuclei were rare, if present at all, but the presence of a few binucleate conidia in such strains made it difficult to determine unequivocally whether the rare conidial-derived cultures, capable of growth on minimal medium, originated from disomic nuclei or heterokaryotic conidia. In spite of the known heterokaryotic nature of PWT cultures,

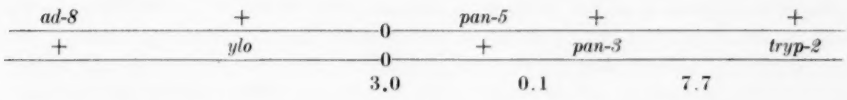
convincing evidence[1-3] has been presented regarding the disomic origin of many PWT strains of which a detailed analysis has been made.

Although we shall continue to refer to PWTs as heterokaryons originating as heterozygous disomic nuclei, the origin of some PWTs may still be questioned. For example, many laboratories use the wild-type strains *74A* and *73a* isolated by St. Lawrence. It is our understanding that one of the reasons these strains were selected was because they were free of chromosome aberrations and because aborted spores in crosses involving such strains were nearly absent. Many investigators working with mutants induced in these wild types have found that the frequencies of PWTs in such crosses were abnormally high in comparison to earlier reports,[1, 2] and often were as great as one per cent, or even greater.[4] Dr. Mary Case, who noted this in crosses involving complementing allelic mutants at the *pan-2* locus, first called it to our attention several years ago. The high proportion of PWTs in the progeny of various crosses involving mutants induced in *74A* leads one to expect a correspondingly high frequency of asci with some aborted spores if the PWTs originated by nondisjunction. However, cursory examination fails to disclose a high enough frequency of such asci with two or four aborted spores to account for the large number of PWTs found in the progeny of such crosses. This suggests that not all strains classified as PWT need have the same origin and that various types of aneuploids may have properties characteristic of PWTs, e.g., heterozygosity of certain loci. That this indeed may be the case was first suggested to us in 1960 by Dr. Mary Case (personal communication), who had evidence of aneuploid types isolated from asci with eight viable spores. Since this manuscript was first prepared, a report by Threlkeld[5] regarding 5:3 asci has appeared which also indicates that aneuploids, possibly in the form of telocentric chromosomes, can be recovered from asci with eight viable spores. Such cases (since they involve aneuploidy and heterozygosity of certain alleles, although they do not involve duplication of entire chromosomes) would have some of the properties of PWT cultures, and might account in part for the high proportion of the aneuploid types recovered from crosses involving *74A*.

In the experiments reported below, it was our original intent to obtain information on whether the homokaryons recovered from PWTs involving linked mutants were largely noncrossover types, as might be expected if they had failed to pair during meiosis and had gone to the poles at random. In preliminary experiments the expected parental genotypes were found, but in some cases nonparental types were also recovered from the same PWT culture. These observations suggested the possibility of somatic recombination. Since PWTs undergo some type of somatic reduction to form a heterokaryon, it is conceivable that the homologous chromosomes of the disomic pair and disjoin in the process of haploidization. The presence of the recombinant is most economically ascribed to somatic crossing over during this process, although other explanations are possible. To obtain further information in support of these findings, a more detailed analysis was made of PWT cultures isolated from crosses between complementary allelic mutants with linked markers. The analyses of a limited number of homokaryotic conidia from 38 different PWT strains have revealed the presence of recombinant types in eight of the strains examined. Reciprocal crossover types were found in three of the eight

PWTs. A detailed examination was made of only five of the strains and these results are reported below.

Materials and Methods.—The pseudowild-types in this investigation were recovered from a cross of *ad-8 pan-5 a* × *ylo pan-3 tryp-2 A*. All of the mutants are located on linkage group VI and the two *pan* mutants are complementing alleles of the *pan-2* locus.[4] In heterokaryotic combinations these alleles are capable of growing on minimal medium, but at a reduced rate. The *ad-8 pan-5* parent was obtained from a cross of *ad-8 ylo A* (FGSC-No. 448) × *pan-5 a*, an isolate received from Dr. Mary Case. The *ylo pan-3 tryp-2 A* isolate also was obtained from the Fungal Genetics Stock Center, Stock Number 469. The location of the mutants is shown on the linkage map below, and approximate distances separating the genes *ylo*, *pan*, and *tryp* are taken from the data of Case and Giles.[4] *ad-8* is located far out on the left arm of the same chromosome but the exact distance from the centromere is unknown.[6] Westergaard's synthetic medium was used



throughout the experiments, and platings of all conidia were done on media supplemented with 0.02% glucose and 1% sorbose in addition to the necessary growth factors.

In the experiments discussed below, single phenotypically wild-type germinating ascospores were isolated and identified as PWTs by their slow growth on minimal medium (characteristic of *pan-3* + *pan-5* heterokaryons) and by differences in the number of colonies formed when conidia of each were plated on minimal and supplemented media. Conidial-derived colonies from supplemented media were then isolated and the phenotype of each was determined by checking its growth requirement. In a preliminary analysis of PWT-18, two conidial-derived cultures were found that required only adenine for growth. Although subsequent analysis of these two adenine-requiring isolates showed them to be heterokaryons between the parental type, *ad pan-5*, and an *ad pan-3 tryp* recombinant, it was, nevertheless, their unexpected presence that first suggested that somatic recombination might occur in PWTs and that prompted a more detailed analysis of this PWT culture. PWT-18, together with PWT-19, selected originally as a control culture but which also proved to have somatic recombinants, were then plated on medium supplemented with adenine sulfate, calcium pantothenate, and tryptophan. After colonies had formed on such plates, approximately 400 colonies from each PWT were then individually transferred to agar slants containing medium supplemented with both pantothenate and tryptophan. The isolates which grew on such medium are called Group I isolates and could be of the phenotype *pan tryp*, *pan*, *tryp*, or heterokaryons. The phenotype of each Group I isolate was further identified by growing each individually on minimal medium as well as tryptophan-supplemented and pantothenate-supplemented liquid media. The colonies that failed to grow on the doubly supplemented pantothenate-tryptophan agar slants within two days (Group II isolates) were then transferred to slants supplemented with pantothenate and adenine. Colonies that still did not show growth (Group III isolates) were again transferred to slants with media triply supplemented with adenine, pantothenate, and tryptophan. Group II isolates that grew on adenine-pantothenate media would be expected to be *pan ad*, or *ad*, isolates or certain heterokaryotic types, and all were further identified by individual testing on pantothenate- and on adenine-supplemented liquid media. Group III isolates were expected to be recombinants of the genotype *pan ad tryp*. This kind of procedure in a modified form has been used routinely in this laboratory as a rapid method of isolating homokaryotic strains from heterokaryons plated on supplemented media. Such colonies maintain their viability over long periods which allows them to be successively transferred from one supplemented slant to another until a supplement is found that satisfies their nutritional requirement. Obviously such tests are not conclusive in themselves, but, coupled with the observation of the morphological mutant, *ylo*, this method serves to pinpoint quickly possible recombinant types. Presumed recombinants were then thoroughly screened by determining their growth requirements in 250 ml Erlenmeyer flasks in shake cultures with 50 ml of appropriately supplemented media. The *tryp-2* mutant is leaky and will grow in the absence of tryptophan after a lag period, which causes some difficulty in routine classification, but such types always were carefully checked

by appropriately timed experiments in critical cases. Some presumed recombinants were further tested to determine whether they were heterokaryotic by plating conidia on several different types of supplemented media, as well as by outcrossing to standard wild-type strains and further checking the mutant progeny. The *pan-3* and *pan-5* alleles were distinguished in heterokaryon tests on agar slants and, when necessary, growth rates were measured in growth tubes of minimal medium at room temperature, or in liquid shake cultures. Heterokaryons between pan-3 and *pan-5* are temperature sensitive and require pantothenate at 35°C which simplifies their identification. However, under any conditions, except when pantothenate is supplied, the growth rate of such heterokaryons is only a fraction of wild-type rates.

Results.—The first stage of analysis of PWTs 18 and 19 consisted of determining the growth requirements of over 800 single conidial isolates. Isolates with the same apparent growth requirements were further subdivided according to the presence or absence of the *ylo* marker, and heterokaryon tests were then carried out to determine the *pan* allele present in selected isolates from each group. In PWT-18 the mutant phenotypes were as follows, with the number of each isolate given in parentheses: *ad pan-5*(119), *ylo pan-3 tryp*(188), *ad*(12), *ylo pan-5*(7), *ad pan-3 tryp*(57), *pan-5*(1), *pan-3 tryp*(33); and 49 isolates grew on minimal and were presumably heterokaryons between the two parental types. From PWT-19 the following types were recovered: *ad pan-5*(108), *ylo pan-3 tryp*(135), *ad*(17), *ad pan-3 tryp*(42), *pan-3 tryp*(17); and 71 heterokaryons grew on minimal. Those isolates with a requirement for adenine as well as the *pan-3 tryp* isolates would have required multiple crossovers to account for their origin by recombination, but they could also be accounted for by certain nuclear combinations in the form of a heterokaryon. Further analyses of such isolates by plating conidia on various supplemented media or by crossing to standard wild-type strains indeed showed that the selected isolates tested were all heterokaryons. Those with a requirement for adenine were heterokaryons between the parental *ad pan-5* and the recombinant type *ad pan-3 tryp*. The *pan-3 tryp* isolates were heterokaryons between the recombinant *ad pan-3 tryp* and the parental *ylo pan-3 tryp*. The single *pan-5* isolate also proved to be a heterokaryon between the parental *ad pan-5* and the *ylo pan-5* recombinant. At present only the *ylo pan-5* and *ad pan-3 tryp* isolates appear to be bona fide recombinants.

In addition to the above analyses, three additional PWTs have subsequently been studied in some detail, and the number of parental and recombinant types identified were as follows: PWT-15—*ylo pan-3 tryp*(153), *ad pan-5*(157), and *ad pan-3 tryp*(7); PWT-24—*ylo pan-3 tryp*(157), *ad pan-5*(81), *ad pan-3 tryp*(41), and *ylo pan-5*(25); and PWT-32—*ylo pan-3 tryp*(108), *ad pan-5*(229), *ad pan-3 tryp*(4), and *ylo pan-5*(4).

Recombinant type *ylo pan-5*, in addition to the two parental types, has been recovered from PWT Nos. 25, 26, and 33, but only a limited number of isolates have yet been tested in these cultures. Of the eight cultures so far analyzed, three have yielded both *ylo pan-5* and *ad pan-3 tryp* reciprocal recombinant types; from two only the *ad pan-3 tryp* recombinant type was recovered; and from three others only *ylo pan-5* recombinants were found. In all, recombinants have been recovered from 8 (21%) of the 38 PWTs examined, but this is obviously a minimum estimate because only a cursory examination of some of the cultures has been made. At present, the only recombinant types that have been recovered are those in which crossing over occurred within a distance of three crossover units between *ylo* and

pan-5 in the vicinity of the centromere. Other recombinant types may be present but undetected because they are either limited in number or present only in heterokaryotic conidia and still unrecognized.

Discussion.—From PWT cultures isolated as single ascospores from crosses of linked mutants, it has been possible to recover homokaryons with the genotypes of the two respective parents as well as homokaryons representing reciprocal crossover products. We have assumed that these recombinants originated from somatic crossing over in the disomic nucleus. Such data suggest that haploidization of the disomic nucleus involves pairing of the homologous chromosomes and that it is during this process that crossing over occurs. It is recognized, however, that the mitotic crossing over and the haploidization need not result from the same mitotic event. Since the mitotic events responsible for haploidization of diploids in Aspergillus[7] and disomics in Neurospora may be quite different, the details of such events in the present examples can only be speculated upon, and additional information is necessary before a complete interpretation of the situation in Neurospora is possible.

The significance of certain of the present findings is not yet clear. Although only a few PWTs with recombinants have been analyzed, it was not anticipated that all of the recombinants would be of the same type and would occur in regions immediately adjacent to the centromere. There is evidence in both Aspergillus[7] and Drosophila[8] for an increased frequency of mitotic recombination in the proximal regions of the chromosome, but to find that all of the observed recombinants occurred within a region of approximately 3 crossover units was not expected. Such results may mean that the pairing of the homologues was confined to the regions immediately adjacent to the centromere and thus the opportunity for exchange was confined to this region. It is hoped that an analysis now in progress with additional markers will provide more information on this question.

The failure always to recover reciprocal crossover products may or may not be significant, especially when one considers the inefficiency of analysis imposed by the heterozygotic effect of heterokaryotic conidia. One may question, nevertheless, whether these differences have any real meaning in terms of the mechanism of formation of recombinant types. For example, one might interpret the difference in the frequency of the reciprocal types, and particularly the failure to recover one type often, to mean that the recombinants were formed nonreciprocally during separate mitotic events. While this may be true, the data are not incompatible with the notion that reciprocal recombinants are initially formed, but that either subsequent events during vegetative growth of the culture result in their unequal frequency, or the observed differences merely reflect the technical problem of identifying rare genotypes in populations of largely heterokaryotic conidia. However, in view of the recent results of Threlkeld[5] concerning the aneuploidy associated with nonidentical sister spores, it is apparent that some heterozygous cultures have different origins and these may behave differently. Since many of his examples appear to involve telocentric chromosomes or centric fragments, whereas the recombinants reported above all have markers on both chromosome arms, it appears that his results are probably not relevant to the present findings.

It has been suggested that the present data do not exclude the possibility that these results occur as a consequence of some aberrant meiotic events rather than

mitotic recombination. For example, if nondisjunction occurred in the first and second divisions successively, after crossing over had occurred, one could obtain tetrasomic PWTs which later might break down into the observed heterokaryon. It is difficult, on the basis of the present evidence alone, to distinguish unequivocally between these two possibilities, especially since the PWTs were isolated as random spores and not from asci. However, the pattern of aborted spores in asci, from which a tetrasomic PWT and a disomic PWT could be recovered, would be different, and a distinction between these two possible origins may eventually be made on this basis. At the same time, the limited data available appear to be more consistent with the somatic recombination hypothesis. In every case so far tested, the homokaryotic conidia of the two parental types were more frequent than the recombinant types, and this would be expected on the assumption that such strains originated as disomics followed by somatic crossing over in some of the disomic nuclei. In these cases it is assumed that only the parental genotypes are present in the original disomic PWT nucleus. The two nuclei within the disomic ascospore then undergo an undetermined number of divisions before haploidization occurs; and either during such divisions or during the division in which haploidization occurs, the somatic recombination may take place. Since recombination would not be expected to take place in every disomic nucleus, then the haploid parental types would always be more frequent than the recombinants. However, in the proposed tetrasomic PWT the constitution of the nuclei within the ascospore would be quite different, since it is assumed that they already contain two parental and two recombinant chromosomes. Regardless of how this complex undergoes haploidization, one would not expect that in every case the nuclei of the parental genotypes would be more frequent than the recombinant types. Furthermore, if a tetrasomic PWT were involved, it is difficult to see why the only recombinants recovered would be those involving exchange in the vicinity of the centromere, unless such recombinant types were in some way predisposed to undergo two successive nondisjunctions, and this seems unlikely if nondisjunction is related to lack of exchange pairing.[9] On the other hand, there is precedence for increased mitotic crossing over in the vicinity of the centromere.[7, 8]

Since PWTs are known to become heterokaryotic, the possibility must also be considered that the present results represent crossing over in diploid nuclei formed by nuclear fusion in the heterokaryon. Such phenomena are known in other ascomycetes, such as Aspergillus,[10, 11] and a single case of apparent recombination of nonlinked genes in a heterokaryon of Neurospora has been attributed by Weijer and Dowding[12] to crossing over between nuclear filaments. During the past several years extensive tests have been carried out in this laboratory using a variety of methods including some which involved highly selective techniques, and we have consistently failed to obtain any unequivocal evidence for either somatic crossing over or reassortment of nonlinked genes in Neurospora heterokaryons. We have also used bisexual heterokaryons in such experiments hoping to get some nuclear fusion in the heterokaryon, but the results were always negative. Our results have never been published, but Pateman and Fincham[13] and more recently Case and Giles[14] have also reported no evidence for somatic recombination in Neurospora heterokaryons.

Since the present results indicate that somatic crossing over is possible in Neuro-

spora, provided a source of aneuploidy is present, we have interpreted the consistent failure to find evidence of somatic crossing over in heterokaryons to indicate that there is no source of aneuploidy present in these cultures. This suggests that fusion of nuclei of the same mating type within a heterokaryon to form diploid nuclei simply does not occur in heterothallic Neurospora. It is for this reason that we favor the hypothesis that it is somatic crossing over in the disomic nucleus that accounts for the results, rather than nuclear fusion within a heterokaryon to give a diploid followed by crossing over and haploidization.

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*INCORPORATION OF 5-IODODEOXYURIDINE INTO DNA OF PLANT CELLS**

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These studies were undertaken to investigate incorporation of the thymidine analogue, 5-iododeoxyuridine (IUdR), into the DNA of higher plant chromosomes.[1] This deoxyriboside was traced in root tip cells of *Vicia faba* (Broad bean) by means of a tritium label; and evidence for incorporation of the iodinated analogue was obtained by use of I^{131} labeled IUdR and cesium chloride density gradient centrifugation of extracted native DNA. IUdR is known to be incorporated into the DNA of microorganisms and mammalian cells,[2] and to increase sensitivity to radiation injury.[3, 4] The base analogue, 5-iodouracil, induces mutation in microorganisms.[5] Special interest is attached to the effect in higher plants of DNA precursor analogues owing to their influence on growth,[6, 7] morphogenesis, and flowering,[8–12] as well as radiosensitization[13] and possibly mutation.[14] Proof of incorporation in higher plant chromosomes remains to be presented and is pertinent to further studies since,

e.g., *Neurospora crassa* appears to lack the thymidine phosphorylating mechanism[15] and thus cannot utilize either thymidine or its analogues, including IUdR. Furthermore, in mammalian tissues the inhibitory effect of IUdR, or its phosphorylated derivatives, is exerted at specific primary metabolic reaction sites which differ according to cell type.[16]

Materials and Methods.—Tritium-labeled IUdR was prepared by iodination of a tritiated sample of deoxyuridine purchased from the New England Nuclear Corporation. Iodination was accomplished by heating for two hr at 60°C a mixture containing 0.50 cc tritiated deoxyuridine (500 μc, 4160 mc/mM), 0.08 cc iodine saturated water, 5.32 cc distilled water, and 5.90 cc 2 *M* nitric acid. After heating, the reaction mixture was neutralized with 1 cc concentrated ammonium hydroxide and adsorbed onto a column of Dowex-1 resin in the acetate form. Washing the column with water removed about 30% of the tritium, presumably in the form of tritiated water since all the activity was volatile. Washing with ammonium acetate buffer (pH 9.0, ionic strength 0.10) removed most of the remaining activity apparently in the form of unreacted tritiated deoxyuridine. Washing with 0.01 *M* acetic acid eluted an amount of activity representing about 5% of the original tritium activity. On the basis of its ultraviolet absorption spectra and the fact that all the activity cocrystallized with carrier IUdR, all the activity in this fraction was considered to be present as tritiated IUdR. The specific activity (calculated from the ultraviolet absorption) of the tritiated IUdR was only 1200 mc/mM, indicating that over 70% of the tritium was lost from deoxyuridine during the course of iodination.

For autoradiographic studies the amount of tritium used in the treatment solutions was 0.86 μc/ml, and the concentration of IUdR (labeled and unlabeled) was 0.72×10^{-6} *M*. Secondary roots of *Vicia faba* grown in half strength Hoagland's nutrient solution were treated for 24 hr with the solution of the tritiated deoxyriboside analogue in deionized water. After treatment the roots were washed in water and transferred to nutrient solution for further growth. All treatment and recovery solutions were aerated and kept at 20°C. Collections of roots were made at three time intervals: the first immediately after treatment, the second 24 hr later, and the third 48 hr after treatment. The 24 hr interval under these conditions is approximately equivalent to one complete division cycle. The roots were fixed in ethanol-acetic acid (3:1), embedded in paraffin, and sectioned longitudinally at 4 microns. The sections were divided into three groups for hydrolysis: no hydrolysis, 12 min, and 90 min in *N* HCl at 60°C. The slides were dipped in liquid nuclear track emulsion (NTB), exposed for from three to seven days, developed, and stained with Delafield's hemotoxylin. Grains in the film, resulting from beta ray emission from H^3 disintegrations, were counted over both the nucleus and cytoplasm of interphase cells in meristematic tissue.

Another group of bean roots was incubated under the conditions described above for 48 hr in a 10^{-3} *M* solution of I^{131} labeled IUdR having a specific activity of 10 mc/mole. The root tips were ground in liquid nitrogen and homogenized in a Waring blendor for two min with 10 cc ice-cold 10% NaCl per gm of root tips. The homogenate was shaken overnight at room temperature with an equal volume of 88% phenol plus $^1/_{10}$ volume 20% KAc. The phases were separated by centrifuging and two volumes of 95% ethanol were added to the clear aqueous layer to precipitate the DNA. This crude DNA was dissolved in 2% KAc, and RNA (but no DNA) was precipitated by making the solution 0.7 *M* in $MgCl_2$. The supernate was twice precipitated with two volumes 95% ethanol and twice redissolved in 2% KAc (Zubay[17]).

To determine the buoyant density of DNA extracted from the IUdR-exposed roots, still another group of beans was grown for 24 hr in the presence of 2×10^{-4} *M* IUdR, both with and without the simultaneous addition of 5-fluorodeoxyuridine (FUdR) at 1×10^{-7} *M*. Frozen root tips were pulverized in a glass homogenizer chilled by dry ice, suspended in 0.15 *M* NaCl + 0.015 *M* Na_3 citrate, and lysed by addition of sodium lauryl sulfate (3%). Deproteinized and RNA-free[18] samples of both the normal and the presumably IUdR-labeled DNA were subsequently centrifuged in the CsCl density gradient using the analytical Spinco ultracentrifuge.[19]

Results and Discussion.—Table 1 shows the average grain count in autoradiographs of root tip cells following nine different treatments. Each figure in the table represents the mean count for approximately 900 interphase cells, i.e., 100

TABLE 1

GRAIN COUNTS IN ROOT TIP CELLS OF *Vicia faba* TREATED FOR 24 HR WITH TRITIATED 5-IODODEOXYURUDINE

Recovery hr	Hydrolysis min	Exposure days	Background	Cytoplasm	Nucleus	Grains/nucleus corrected for 7-day exposure
0	0	3	0.99	0.90	5.29	12.33
0	12	3	0.73	1.00	4.57	10.65
0	90	3	0.76	0.61	2.93	6.83
24	0	5	0.78	0.84	5.13	7.18
24	12	5	0.93	0.81	4.35	6.09
24	90	5	0.87	0.62	3.09	4.33
48	0	7	0.75	0.43	3.17	3.17
48	12	7	0.63	0.40	2.35	2.35
48	90	7	0.99	0.81	1.39	1.39

from each of three mid-longitudinal sections on each of three separate root tips. The "background" per cell area was determined by grain counts over enucleated cells. It is evident from the table that there were no more grains over the cytoplasm than "background" level and, therefore, that no incorporation of tritium label has taken place in the cytoplasm. It is equally evident that there has been substantial incorporation in the nucleus (Table 1). Examination of dividing cells shows clearly that this incorporation is selectively in the chromosomes (Fig. 1*A*). No grains were observed over the nucleolus (Fig. 1*B*). This evidence, together with the lack of cytoplasmic label, substantiates the conclusion that neither the IUdR nor tritium exchange products were incorporated into cytoplasmic or nucleolar RNA or other cytoplasmic components such as proteins.

Hydrolysis for 12 min with hot *N* HCl, which removes the RNA, most of the purines of DNA, and probably some DNA pyrimidines and proteins, resulted in a 14% reduction in grain counts over the nucleus in those slides fixed immediately after treatment with the tritiated analogue. Hydrolysis for 90 min would be expected to remove all nucleic acids (RNA and DNA)[20] leaving mostly proteins as

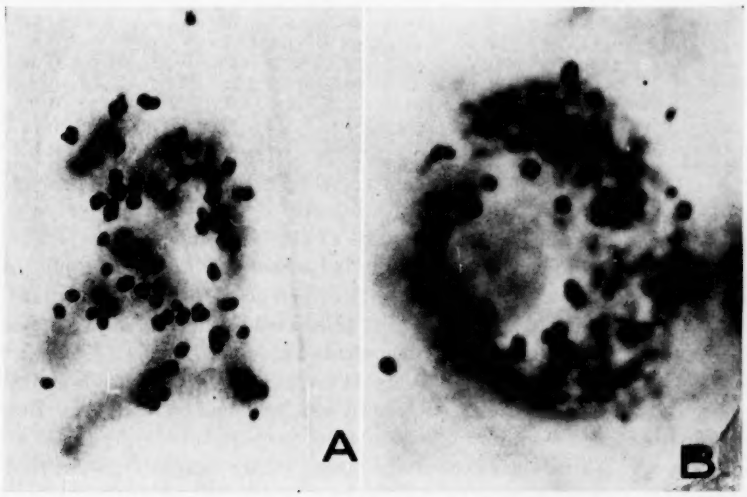

FIG. 1.—Nuclei labeled with tritiated IUdR: (*A*) showing incorporation in metaphase chromosomes, (*B*) showing lack of incorporation in resting stage nucleolus.

potentially labeled cellular compounds. After this prolonged hydrolysis there were 45% fewer grains than over unhydrolyzed nuclei. The substantial additional reduction in labeling (31%) associated with the 90 min compared to 12 min hydrolysis is attributable largely to removal of DNA pyrimidines. This evidence confirms, therefore, the expectation that the analogue replaces thymidine in Vicia chromosomes. The appreciable amount of label remaining after 90 min hydrolysis, i.e., 55, 60, and 44%, respectively, following 0, 24, and 48 hr recovery periods, indicates either incomplete hydrolysis of DNA or that a portion of the label is taken up by nuclear proteins.

For each cell division cycle, expected to occur on an average in 24 to 30 hr[21] following incorporation of the tritiated analogue, there should be a halving of the amount of label per nucleus. In the last column of Table 1, the grain counts per nucleus are compared on a common basis of 7-day exposure time. The differences between counts after 12 and 90 min hydrolysis are the best estimates of amounts of labeled pyrimidine. These values after 0, 24, and 48 hr recovery are 3.82, 1.76, and 0.96, respectively. The counts are approximately halved after 24 hr and are about one fourth the original amount per nucleus after 48 hr. These results show that on an average DNA synthesis and the division cycle are not detectably affected according to this criterion during the first two division cycles after incorporation. No evidence of chromosome breakage was found.

A more detailed presentation of the results on grain counts over nuclei, which were summarized in Table 1, is shown in Table 2. A large range in numbers of grains over

TABLE 2

DISTRIBUTION OF GRAIN COUNTS IN NUCLEI OF *Vicia faba* ROOT TIP CELLS TREATED WITH TRITIATED 5-IODODEOXYURIDINE

Total cells	Recovery hr	Hydrolysis min	Per cent nuclei with grain counts of 0–2	3–5	6–8	9–11	12–14	15–17	18–20	20+
901	0	0	35.5	27.0	17.1	8.4	5.8	3.9	1.1	1.2
900	0	12	37.6	31.0	17.3	6.2	4.9	1.6	0.89	0.44
892	0	90	51.0	29.4	13.5	3.8	1.2	0.45	0.00	0.11
		$\bar{x}$	41.4	29.3	16.0	6.1	4.0	1.98	0.66	0.58
900	24	0	48.0	19.8	11.9	6.1	5.2	3.2	2.0	3.8
900	24	12	48.1	22.6	13.2	8.0	4.1	1.7	0.44	1.9
900	24	90	59.2	21.6	10.3	4.8	2.7	0.67	0.44	0.33
		$\bar{x}$	51.8	21.3	11.8	6.3	4.0	1.86	0.96	2.01
900	48	0	60.2	21.0	8.9	4.3	3.0	1.1	0.67	0.78
892	48	12	70.1	17.5	7.1	1.6	2.6	0.45	0.22	0.56
889	48	90	80.7	16.8	2.1	0.34	0.11	0.00	0.00	0.00
		$\bar{x}$	70.3	18.4	6.03	2.08	1.90	0.52	0.30	0.45

nuclei is observed. The varying amounts of labeled analogue incorporated into root tip cells may be due to: (1) differences between cells in time spent in DNA synthesis during the treatment period, (2) differences in availability of compound in different regions of the root tip, (3) differences in rate of DNA synthesis in different cell types, and (4) differences in size of nucleus and plane of sectioning through the nucleus. Following 24 and 48 hr recovery periods there is a decrease in the proportion of densely labeled cells, as would be expected. However, a small number of cells with a high grain count is characteristically found after 48 hr of recovery. This indicates that the more heavily labeled cells fail to divide, either because of the radioactivity, the higher concentration of IUdR, or most likely a combination of the two. Hydrolysis for 12 and 90 min causes a progressive de-

crease in label throughout the range observed. No highly labeled cells remained after 48 hr recovery and 90 min of hydrolysis. The autoradiographic results show that the H^3 label has been incorporated into the chromosomal DNA; however, the label could conceivably have been exchanged to thymidine during cell metabolism before incorporation. In view of the importance in mutagenic action[22] of incorporating the analogue *per se*, the DNA was extracted to test for separation of normal thymidine-containing DNA from the iodinated form.

Analyses of extracted DNA from roots treated for 48 hr in a 10^{-3} *M* solution of I^{131} labeled IUdR having a specific activity of 10 mc/mole gave the following results. The purified DNA solution had an ultraviolet absorption pattern characteristic of nucleic acid. No ultraviolet absorbing impurities could be detected. From the radioactivity of the solution it was calculated that the DNA contained 1.4% IUdR by weight. This corresponds to the substitution of an average of about 5% of the thymidine by IUdR. The equilibrium distribution of this DNA in a cesium chloride density gradient is skewed toward higher densities but no independent band of "heavy" DNA was observed. There appears to be no significant amount of DNA in which more than 15% of the thymidine is replaced by IUdR. This conclusion, based on ultraviolet photographs of the DNA distribution, is confirmed by direct sampling of the density gradient. No radioactivity was found at densities corresponding to more than 20% substitution of thymidine by IUdR. Since the IUdR was considered to be present during a single cell division cycle only, it is to be expected that the DNA will be unifilarly labeled. Therefore, the IUdR-labeled strands have an average of at least 10% of their thymidine replaced by IUdR but none has more than 40% replaced.

The small minimal replacement indicated may be due to adverse effects on cellular metabolism caused by the excessively high concentration of IUdR. Further experiments were performed with roots cultured for 24 hr in unlabeled IUdR at 2×10^{-4} *M*, which is about five times lower than that used in the previous experiments and 200 times more concentrated than was used in the autoradiographic studies.

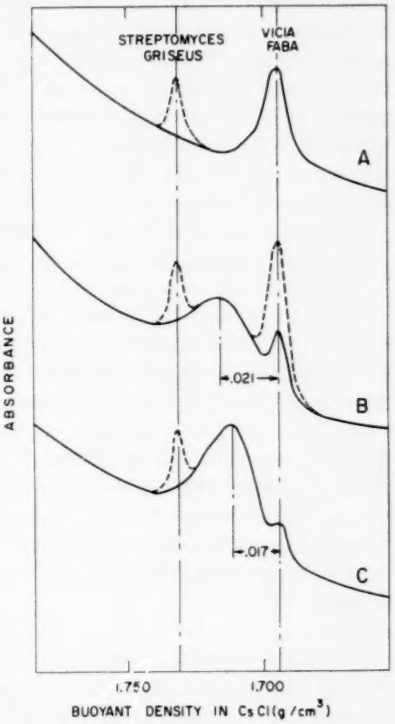

FIG. 2.—Microdensitometer tracings of photographs taken after 20 hr of CsCl-equilibrium-density-gradient centrifugation (44,770 rpm; 25°C) of DNA extracted from normal root tips of *Vicia faba* (*A*) and from roots grown for 24 hr in the presence of 2×10^{-4} *M* IUdR and 10^{-7} FUdR (*B*) or in the presence of 2×10^{-4} *M* IUdR alone (*C*). Note that under conditions *B* and *C*, a preponderant proportion of the DNA acquires a new heavier buoyant density. The density reference peaks (broken lines) were obtained by repeating the centrifugation after addition, directly to the cells, of approximately 1 μg of *Streptomyces griseus* DNA (1.731 g/cm³) or of normal *Vicia faba* DNA (1.696 g/cm³).

Three lots were grown for the 24 hr period: (*A*) in aerated deionized water at 20°C as an untreated control, (*B*) in a solution of 2×10^{-4} *M* IUdR with 1×10^{-7} *M* FUdR, and (*C*) in a solution of 2×10^{-4} *M* IUdR alone.

The DNA, which was extracted and purified according to the methods described above, was centrifuged at 44,770 rpm at 25°C for 20 hr in a CsCl equilibrium density gradient. Microdensitometer tracings of photographs taken after the centrifugation are illustrated in Fig. 2. The results show that when roots are grown in the presence of IUdR, alone or with FUdR, a preponderant proportion of the DNA acquires a new heavier buoyant density. Since exposure to IUdR was of comparatively short duration, this new peak most probably represents unifilarly IUdR-labeled ("hybrid") DNA. The degree of thymidine replacement by IUdR in the IUdR-containing strands, synthesized after addition of IUdR, should correspond to approximately 28% (*B*) or 22% (*C*), as calculated from the shift of the main peak (Erikson and Szybalski,[23] in press). FUdR was added in treatment (*B*) to facilitate uptake of IUdR through inhibition of thymidylate synthetase[4] and, as a consequence, DNA replication in *Vicia faba* root cells.[24] A slightly greater incorporation was indicated, 28% in (*B*) versus 22% in (*C*) which contained IUdR alone. However, the fact that almost as much IUdR was incorporated without the addition of an inhibitor shows that IUdR can replace thymidine in the synthesis of Vicia DNA and is effectively incorporated even in the presence of normal cellular levels of thymidine.

Summary.—Incorporation of 5-iododeoxyuridine into the DNA of root tip chromosomes of *Vicia faba* is demonstrated by: (1) Tritium label introduced with the iodinated analogue is detectable exclusively over nuclei of interphase cells and specifically over chromosomes in dividing cells. (2) No grains are found over nucleoli. (3) The number of grains per nucleus is approximately halved after one division cycle and quartered after two divisions. (4) Incorporation of the iodinated analogue was demonstrated by use of an I^{131} label. (5) CsCl density gradient centrifugation of extracted native DNA, following 24 hr treatment with IUdR, reveals a new heavier component attributable to replacement of thymine methyl groups by iodine. The degree of thymidine replacement by IUdR in the IUdR-containing strands was calculated to be about 22%, and slightly greater (28%) if FUdR was included in the treatment solution.

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*DUALITY FOR COMPACT GROUPS**

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For some time I have conjectured the existence of a duality theory for compact groups which is of the following sort: To each such group G there is assigned a dual algebraic object so that (1) the dual displays the structure of the irreducible unitary representations of G, (2) the dual determines G to a group isomorphism, (3) homomorphisms of groups induce reasonable mappings of their duals, i.e., the duality is functoral, and (4) the class of duals of groups has a reasonably simple algebraic description of its own. Pontrjagin duality does just this for abelian groups, and Tannaka's theorem indicates the probability that (1) and (2) are generally possible, although Tannaka's formulation surely lacks (4). This note outlines a duality which has all four properties.

The dual of a group, which I call a cogroup, is a complex linear space which has both the structure of a *-algebra and that of a co-*-algebra. We first describe the co-*-algebraic structure, then the algebraic, and then the identities which link these. Finally, we sketch the construction of the corresponding group from an abstractly given cogroup.

A last general remark: If G is abelian, the cogroup R of G is just the set of finite linear combinations of characters. This suggests that R may not be the most primitive dual object, but may rather be the analogue of the group algebra of some simpler object. I do not know whether this is the case.

The Co--Algebra* (R, γ, c).—Let R be the linear space of all representative functions on the compact group G. A continuous complex-valued function f belongs to R if the linear space spanned by all left (or right, or left and right) translates of f is finite-dimensional, or, equivalently, if f is a finite linear combination of elements of irreducible unitary matrix representations of G. If $f \in R$, then there are continuous functions $g_1, g_2, \ldots, g_n$ and $h_1, h_2, \ldots, h_n$ so that $f(xy) = \Sigma_{i=1}^{n} g_i(x)h_i(y)$ for all x and

y in G. The functions g_i and h_i are not unique, but setting $\gamma(f) = \Sigma_{i=1}^{n} g_i \otimes h_i$, we find that γ is a linear map of R into the tensor product $R \otimes R$. There is also a natural adjoint operation, defined by setting $a(f)(x)$ equal to the complex conjugate $c_k \circ f(x^{-1})$ of $f(x^{-1})$ for all $f \in R$ and $x \in G$. The structure of (R, γ, a) is very simple; it is the direct sum of finite dimensional sub-co-*-algebras R_α (i.e., $\gamma[R_\alpha] \subset R_\alpha \otimes R_\alpha$ and $a[R_\alpha] = R_\alpha$), and each of these is isomorphic to the dual of the algebra of all linear transformations on a finite dimensional Euclidean space, with γ and a being the duals of composition and the adjoint operation, respectively. (Each irreducible sub-co-*-algebra R_α is precisely the linear combinations of entries of an irreducible unitary matrix representation of G.) The linear functional on R which corresponds to the unit of the dual algebra we denote by e.

It is easy to give other intrinsic characterizations of the structure (R, γ, a), but we shall not do this here. Such a structure (R, γ, a) will be called a *completely reducible co-*-algebra.*

*The *-Algebra (R, μ, c).*—The class R of representative functions of a compact group G is closed under pointwise multiplication and conjugation. Let μ be the map of $R \otimes R$ into R induced by multiplication, and let $c(f)$ be the complex conjugate of f for $f \in R$. The algebra (R, μ, c) can be described as a *semi-simple commutative *-algebra* with unit 1 (that is, for each $f \in R$ with $f \neq 0$ there is a complex-valued homomorphism X of R such that $X(f) \neq 0$ and $X(c(g)) = c_k \circ X(g)$ for $g \in R$, where c_k is complex conjugation).

The Linking Identities.—The following identities connect the algebraic and coalgebraic operations on the space of representative functions.

(1) $\gamma \circ u = (\mu \otimes \mu) \circ F_{23} \circ (\gamma \otimes \gamma)$ *where* $F_{23}(f \otimes g \otimes h \otimes k) = f \otimes h \otimes g \otimes k$.

(2) $\mu \circ (I \otimes c \circ a) \circ \gamma(f) = e(f) \cdot 1$ *all* $f \in R$ *where* I *is the identity map on* R.

(3) $\gamma \circ c = (c \otimes c) \circ \gamma$, $a \circ \mu = \mu \circ (a \otimes a)$ *and* $a \circ c = c \circ a$.

The first of these is a restatement of the fact that $x \to (x, x)$ is a homomorphism of G into $G \times G$, using the isomorphism of $R(G \times G)$ and $R(G) \otimes R(G)$, and the second is derived from the map $x \to (x, x^{-1})$ followed by $(x, y) \to xy$. All are simple to prove by direct computation.

A *cogroup* is a system (R, γ, a, μ, c) such that (R, γ, a) is a completely reducible co-*-algebra, (R, μ, c) is a semi-simple *-algebra with unit, and the linking identities (1), (2), and (3) hold. The set of representative functions of a compact group is, with operations as defined above, a cogroup.

The Group of a Cogroup.—We now outline the construction of a compact group G from an abstractly given cogroup R. We define G to be the set of complex-valued μ-homomorphisms x of R which are c-self adjoint (i.e., $x \circ c = c_k \circ x$) and for which $x(1) = 1$. For $x, y \in G$ the group product xy is defined to be $\mu_k \circ (x \otimes y) \circ \gamma$ where μ_k is complex multiplication. From the linking identity (1) we see that xy is a μ-homomorphism and (3) implies easily that if $x \in G$, then $c_k \circ x \circ a \in G$. Identity (2) is crucial in showing that $x^{-1} = c_k \circ x \circ a$. The restrictions of members of G to irreducible sub-co-*-algebras of R are thus shown to be a group of unitary transformations, and G is compact relative to the product topology. Finally, evaluation carries members of R into representative functions on G, and it can be shown by means of the Stone-Weierstrass theorem and the usual orthogonality relations that R maps *onto* the set of representative functions of G.

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ON SPRAYS AND HIGHER ORDER CONNECTIONS

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The purpose of this note is to formulate the theory of sprays in terms of semi-holonomic jets[1a] (thus we give new proofs of some results obtained by Ambrose-Palais-Singer[2]) and to extend it to higher order connections. Higher order connections have been introduced by C. Ehresmann[1b] but our definition is different and less general.

Details will be given in a paper to appear later.

Notations and definitions: Let V_n and V_m be paracompact differentiable manifolds (by differentiable we always mean C^∞) of dimensions n and m; for any map $f: U \subset V_n \to V_m$, f_* will be the prolongation to tangent vectors. Let $\mathcal{E}(V_n,F,G,\mathfrak{C})$ be a fiber bundle over V_n, with standard fiber F, structural group G, principal bundle $\mathfrak{C}$; if $s \in G$, s will also denote the right translation: $h \to hs$ of $\mathfrak{C}$ onto itself; $T(\mathfrak{C})/G$ will denote the vector bundle obtained from the tangent bundle $T(\mathfrak{C})$ by identifying vectors which are transforms of each other under right translations of G. Let φ be the map: $\mathfrak{C} \times \mathfrak{C} \to \mathfrak{C}\mathfrak{C}^{-1}$ defined by $\varphi(h,h') = h'h^{-1}$; an *infinitesimal displacement*[1c] δ_x of the fiber $F_x (x \in V_n)$ is a vector $\varphi_*(0_h,X_h)$ where 0_h is the zero vector and X_h any vector which is also tangent to $\mathfrak{C}$ at $h \in \mathfrak{C}_x$; the origin of δ_x in the groupoid $\mathfrak{C}\mathfrak{C}^{-1}$ is the identity map of F_x; as $\varphi_*(0_h,X_h) = \varphi_*(0_{hs}, s_*X_{hs})$, the space of all infinitesimal displacements of F_x can be identified with $T(\mathfrak{C}_x)/G$.

Let $J^q(V_m,V_n)$ be the bundle of all q-jets of V_n into V_m, α: $J^q(V_m,V_n) \to V_n$ be the projection sending each jet onto its source; if σ is a local *lifting* of V_n into $J^1(V_m,V_n)$ ($\alpha\sigma$ is the identity map of $U \subset V_n$), the jet $j^1_x \sigma$ will be called *non-holonomic jet* of order 2 of V_n into V_m; let $\tilde{J}^2(V_m,V_n)$ be the space of all these jets. By induction on q, we define $\tilde{J}^q(V_m,V_n)$. If, for any k, the lifting σ of V_n into $\tilde{J}^k(V_m,V_n)$ satisfies: $j^1_x(j^{k-1}\cdot\sigma) = \sigma(x)$ where j^{k-1} is the projection: $\tilde{J}^k(V_m,V_n) \to \tilde{J}^{k-1}(V_m,V_n)$, we get the *semi-holonomic* jets; let $\bar{J}^q(V_m,V_n)$ be the set of all semi-holonomic q-jets; we have: $J^q(V_m,V_n) \subset \bar{J}^q(V_m,V_n)$. The space $\bar{\Pi}^q(V_n)$ (or more briefly $\bar{\Pi}^q$) of *semi-holonomic frames* of order q is a fiber bundle which is associated to $H^q(V_n)$ (space of holonomic frames); moreover $\bar{\Pi}^q$ is a principal bundle, with structural group $\bar{L}^q_n$ (group of all invertible semi-holonomic q-jets of R^n into R^n, with source and target 0). Every $y \in \bar{L}^q_n$ can be written: $\bar{x}^i = \Sigma a^i_j x^j + \ldots + 1/q!\ \Sigma a^i_{j_1}\ldots_{j_q} x^{j_1} \otimes \ldots \otimes x^{j_q}$ where the matrix $a^i_j \in L^1_n$ ($= L_n$ = full linear group); $y \in L^q_n$ if and only if the $a^i_{j_1}\ldots_{j_q}$ are symmetrical. By symmetrization we get a projection P_q of $\bar{\Pi}^q$ onto H^q. Let $\bar{M}^q_n$ be the kernel of the homomorphism j^{q-1}: $\bar{L}^q_n \to \bar{L}^{q-1}_n$; $\bar{M}^q_n$ is an abelian group and its Lie algebra can be identified with $R^n \otimes (\overset{q}{\otimes} R^{n*})$. We have: $\bar{M}^1_{n,n} = M^1_{n,n} = L_{n,n}$ = space of all $n \times n$ matrices. We shall write: $H^0 = V_n$.

Let $S^q_x(V_n)$ (or more briefly S^q_x) be the vector space of all q-jets (with source x) of the local liftings of V_n into the tangent bundle $T(V_n)$ and $S^q = \bigcup_{x \in V_n} S^q_x$; using semi-holonomic jets, we define $\bar{S}^q_x$ and $\bar{S}^q$; we have $S^0_x = T_x$ (tangent space

to V_n at x). A local lifting of V_n into $\bar{S}^q$ is represented in the domain U_α of local coordinates $x^1, \ldots, x^n$ by a sequence $X^i, X^i_j, \ldots, X^i_{j_1 \ldots j_q}$ of functions such that:

$$X^i_{j_1 \ldots j_s + 1} - \frac{\partial X^i_{j_1 \ldots j_s}}{\partial x^{s+1}}$$

are, for any $s \leq q$, the components of a tensor field.

$\bar{S}^q$ is a vector bundle which is associated to the principal bundle $\bar{\Pi}^{q+1}$; the standard fiber is: $R^n \oplus L_{n,n} \oplus \bar{M}^2_{n,n} \oplus \ldots \oplus \bar{M}^q_{n,n} = R^n \oplus \mathfrak{v}^q_n$ (where $\mathfrak{v}^q_n$ is the Lie algebra of $\bar{L}^q_n$); the structural group is $\bar{L}^{q+1}_n$; it can be proved that $\bar{S}^q$ is *canonically isomorphic* to $T(\bar{\Pi}^q)/\bar{L}^q_n$. Thus $\bar{S}^q_x$ can be defined by induction on q: *$\bar{S}^q_x$ is the space of infinitesimal displacements of the fiber $\bar{S}_x{}^{q-1}$ belonging to the bundle $\bar{S}^q$.*

We define an *elementary connection of order r* at $x \in V_n$ as a linear lifting $\bar{C}^r_x$ of $\bar{S}^{r-1}_x$ into $\bar{S}^r_x$; it can be deduced a linear map of $\bar{S}^r_x$ into the tensor space $T_x \otimes (\overset{r}{\otimes} T_x{}^*)$ (our previous definition[5] is equivalent to a lifting of S^{r-1}_x into $\bar{S}^r_x$). A *connection of order r* is a differentiable field $x \to \bar{C}^r_x$ of elementary connections; it is a section of the fiber bundle $\mathfrak{A}^r$ of all elementary connections; the standard fiber $(R^n \oplus L_{n,n} \oplus \ldots \oplus \bar{M}^{r-1}_{n,n})^* \otimes \bar{M}^r_{n,n}$ being homeomorphic to a numerical space, such a section always exists. For $r = 1$, we get the usual affine connection. The following theorem can be proved:

THEOREM. *Every connection $\bar{C}^r$ induces a connection C in the principal bundle $\bar{\Pi}^r \to \bar{\Pi}^{r-1}$ (with structural group $\bar{M}^2_n$) such that the field of horizontal vector subspaces is invariant under the right translations of the group $\bar{L}^r_n$; conversely, C induces $\bar{C}^r$.*

The proof uses the fact that to each $y \in R^n \oplus \mathfrak{v}^{r-1}_n$, the connection $\bar{C}^r$ associates a horizontal vector field $\bar{X}^r$ (or *basic* vector field), which is invariant under the right translations of $\bar{L}^r_n$: if $h_r \in \bar{\Pi}^r_x$, the vector $h_r y$ belongs to $\bar{S}^{r-1}_x$; its lift in $\bar{S}^r_x$ determines a horizontal vector at h_r. We deduce:

COROLLARY. *Every connection of order r on V_n gives rise to a global parallelism on $\bar{\Pi}^r$.*

The principal bundle $\bar{\Pi}^r \to \bar{\Pi}^{r-1}$ is a sub-bundle of $H^1(\bar{\Pi}^{r-1})$ obtained by reduction of the group $L_{n2^{r-1}n}$ to $\bar{M}^2_n$; therefore, C is an *affine connection;* so we can define the notion of *parallel displacement* along a curve of $\bar{\Pi}^{r-1}$; we define the *geodesics of order r:* they are the geodesics of the connection C. The set of all these geodesics is invariant under the translations of $\bar{\Pi}^{r-1}$: $h \to hs$ (where $s \in \bar{L}^{r-1}_n$).

If $r > 1$, the group $\bar{M}^2_n$ being abelian, the *curvature* forms of C are invariant forms on $\bar{\Pi}^{r-1}$ and are the exterior derivatives of forms θ^r_α on $\bar{\Pi}^r$; if C' is another connection, $\theta^r_\alpha - \theta'^r_\alpha$ are forms on $\bar{\Pi}^{r-1}$; so the *curvature forms* define on $\bar{\Pi}^{r-1}$ *cohomology classes which are independent of the connection* (we get a particular case of Weil's theorem).

An elementary connection $\bar{C}^r_x$ defines a structure of Lie algebra on $\bar{S}^{r-1}_x$; the *torsion* of $\bar{C}^r_x$ is the element θ_x of $S_x{}^{r-1} \otimes (S^{r-1}_x \wedge S^{r-1}_x)^*$ defined by: $\theta_x(X^{r-1} \wedge Y^{r-1}) = [X^{r-1}, Y^{r-1}]$ for any $X^{r-1}, Y^{r-1} \in \bar{S}^{r-1}_x$. For $r = 1$, we get the usual torsion.

If $r = 1$, the bundle $\bar{\mathfrak{A}}^1$ of elementary connections (with fiber $\bar{M}^2_n$) can be identified with $\bar{\Pi}^2/L_n$; the projection P^2: $\bar{\Pi}^2 \to H^2$ defines a projection p of $\bar{\mathfrak{A}}^1$ onto $\mathfrak{A}^1$ (bundle of symmetric or torsionless connections) and we get Kobayashi's result.[3]

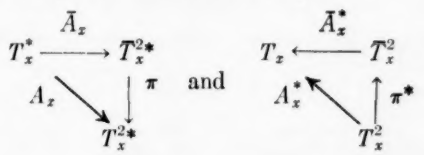
Thus, *every elementary connection* $\bar{C}^1_x \in \bar{\mathfrak{A}}^1$ *is determined by its projection on* $\mathfrak{A}^1$ *and its torsion.*

An infinitesimal displacement δ_x of T_x is a lifting of T_x into $\mathfrak{I}_x$, space of all tangent vectors to $T(V_n)$ whose projection on $T(V_n)$ and origin are the same element of T_x. Any local coordinates system defines an isomorphism of $\mathfrak{I}_x$ onto $T_x \times T_x$ and associates to δ_x an element of $T_x \otimes T^*_x$; so to the elementary connection $\bar{C}^1_x \in \bar{\mathfrak{A}}^1$, there corresponds an element of $(T_x \otimes T^*_x) \otimes T^*_x$ (which is a bilinear map of $T_x \times T_x$ into T_x), and we get the Christoffel symbols. If δ_x is the infinitesimal displacement image of $X \in T_x$ by the linear map $\bar{C}^1_x$, then δ_x lifts any $Y \in T_x$ into $[Y,(dY/dt)] \in \mathfrak{I}_x$ such that: $dY/dt = b(X,Y)$ where b is bilinear; in particular $(dX/dt) = Q(X)$ where Q is the quadratic form associated to b; for all elementary connections which project on the same symmetric elementary connection we get the same quadratic form; $[X, (dX/dt)]$ is a 2-*velocity* with origin x (it is a 2-jet of R into V_n with source 0 and target x). *We define an elementary spray* Σ_x *at* x *as a lifting of* T_x *into* ${}^2T_x$ *(space of all 2-velocities at* x*) such that for any local coordinates,* $(d^2x/dt^2) = Q(dx/dt)$ *where* Q *is a quadratic form.* A spray[4] is a differentiable field $x \to \Sigma_x$; when it is induced by a connection, its integral curves are the geodesics.

Let Σ be a local spray (defined in an open set $U \ni x$) such that $\Sigma(x) = \Sigma_x$ and let $f_v(t)$ be the solution of Σ corresponding to the initial conditions: $f(0) = x$, $(df_v/dt)_{t=0} = v$. We define $\exp_{x,\Sigma}$: $\mathcal{O}_\Sigma \to V_n$ (where $\mathcal{O}_\Sigma$ is an open neighborhood of 0_x in T_x) by: $\exp_{x,\Sigma}(v) = f_v(1)$. It can be proved that the 2-jet $j^2_{0x} \exp_{x,\Sigma}$ (whose projection is the 1-jet of the identity map of T_x) depends only on the elementary spray Σ_x and define a symmetric elementary connection (its Christoffel symbols are the second order derivatives of the x^i's with respect to the components of $v \in T_x$). So we get:

THEOREM. *All elementary connections at* x *which project on the same symmetric connection give rise to the same elementary spray. Conversely, every elementary spray* Σ_x *generates an elementary symmetric connection defined by the jet* $j^2_{0x} \exp_{x,\Sigma}$, *where* Σ *is any local spray such that* $\Sigma(x) = \Sigma_x$.

There is a (1,1) correspondence between linear liftings $\bar{C}^1_x$ of T_x into S^1_x and linear liftings $\bar{A}_x$ of T^*_x into T^{2*}_x, space of 2-covelocities (or semi-holonomic 2-jets of V_n into R, with source x and target 0). By duality we get a projection $\bar{A}^*_x$ (transpose of $\bar{A}_x$) of T^2_x (dual of T^{2*}_x) onto T_x. If A_x is the linear lifting corresponding to the symmetric connection $p(\bar{C}^1_x)$ and π the projection of T^{*2}_x onto T^{*2}_x (space of holonomic 2-velocities), we have the commutative diagrams:



where T^2_x (dual of T^{2*}_x) can be called the space of second order tangent vectors[2]; thence:

PROPOSITION. *Every elementary connection* $\bar{C}^1_x$ *defines a projection of* T^2_x *onto* T_x, *whose restriction to the space* T^2_x *of second order tangent vectors is determined by the projection* $p(\bar{C}^1_x)$ *of* $\bar{C}^1_x$ *onto the space of symmetric connections.*

The elementary connection $\bar{C}^1_x$ can also be defined as a R-linear map ∇_x of the space of germs at x of Pfaffian forms into $T^*_x \otimes T^*_x$, such that for every local co-

ordinates x^i, we have $(\nabla_x(a))_{ij} = (\partial a_i/\partial x^j)_x$ for any a satisfying: $a(x) = 0$. The elementary connection is torsionless if and only if the tensor $\nabla_x a$ is symmetric for any a. If $p\nabla_x$ corresponds to the symmetric connection $p(\bar{C}_x^1)$, for any differentiable function defined near x, $p\nabla_x(df)$ will be called the *hessian* of f at x.[2] From our study, we deduce: *There is a* (1.1) *correspondence between hessians and elementary sprays at* x.

A spray Σ^r *of order* r on V_n is defined as a spray Σ on $\bar{\Pi}^{r-1}$ satisfying the condition: $\exp_{h,\Sigma}(v)$. $s = \exp_{hs,\Sigma}(s_* v)$ for every $h \in \bar{\Pi}^{r-1}$, $s \in \bar{L}_n^{r-1}$, $v \in T_h(\bar{\Pi}^{r-1})$ such that the relation has a meaning. The geodesics of a connection $\bar{C}^r$ determine a spray. Conversely, given a spray Σ^r, the map $\exp_{h,\Sigma}$ induces at every $x \in V_n$ a map ψ_x of $\mathfrak{U}$ (open neighborhood of 0_x in $\bar{S}_x^{r-1}$) onto V_n and the jet $j_{0x}^{r+1}\psi_x$ determines an holonomic elementary connection of order r (this connection lifts S_x^{r-1} into S_x^r): its "Christoffel symbols" are the $(r+1)^{\text{th}}$ derivatives of the x^i's with respect to some coordinates in $\bar{S}_x^{r-1}$.

Remark: The kernel $S_x'^1$ of the projection; $S_x^1 \to T_x$ is canonically isomorphic to $T_x \otimes T_x^*$; so in particular the identity map $\tilde{x}$ of T_x belongs to $S_x'^1$; its lift by any elementary connection $\bar{C}_x^2$ of order 2 is an element of the fiber over x of the bundle $\bar{\Pi}^2/L_n$; thence:

THEOREM. *Every elementary connection* $\bar{C}_x^2$ *of order* 2 *determines an elementary connection of order* 1. (This theorem has been proved by computation in ref. 5.)

The notion of higher order connection can be extended to G-structures, that is, a reduction of L_n to a Lie subgroup G; we then consider the principal bundle $\bar{\Pi}'^2$, with structural group $\bar{G}_r \subset \bar{L}_n^r$ (semi-holonomic prolongation of G).

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ALMOST AUTOMORPHIC FUNCTIONS

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S. Bochner has introduced in various contexts the notion of almost automorphy.[1-3] The results of the present note are stated for the integers and real line, although on a future occasion we shall deal with a more general setting. (We adopt the abbreviations of ref. 3.)

Definition 1 (Bochner): A function $f(t)$ on an abelian group G is a.a.(= almost

automorphic) if every seq. $\{\alpha'_n\} \subset G$ contains a subs. $\{\alpha_n\}$ for which both

$$\lim_{n\to\infty} f(t + \alpha_n) = g(t) \tag{1}$$

and

$$\lim_{n\to\infty} g(t - \alpha_n) = f(t) \tag{2}$$

hold for any $t \epsilon G$ but *not necessarily uniformly.*

The question was raised[3] whether the functions of Definition 1 are more general than the a.p.(= almost periodic) functions of Bohr. The following construction for $G = Z =$ integers will show that this is indeed the case.

Let $G_1, G_2, \cdots$ be a seq. of inf. subgroups of Z such that

(i) G_1 is proper in Z.
(ii) G_{k+1} is proper in G_k for each k.

Associated sequences of integers $\{a_k\}$ and sets $\{A_k\}$ are defined inductively as follows. Let $a_1 = 0$, $A_1 = G_1$. By (i) $A_1 \neq Z$. Having defined $A_k = a_k + G_k$ for $k = 1, 2, \cdots, n$ such that $\bigcup_{k=1}^{n} A_k \neq Z$ and $A_p \cap A_q = \phi$, $p \neq q$, we take a_{n+1} to be an integer of minimum modulus which is not in $\bigcup_{k=1}^{n}$. Set $A_{n+1} = a_{n+1} + G_{n+1}$. Using property (ii) it is easily verified that

(a) $A_{n+1} \cap A_k = \phi$, $k = 1, 2, \cdots, n$

(b) $\bigcup_{k=1}^{n+1} A_k \neq Z$.

By our choice of a_{n+1} the seq. $\{A_k\}$ will satisfy in addition

(c) $\bigcup_{k=1}^{\infty} A_k = Z$.

We take a seq. $\{f_k\}$ of functions such that f_k is for each k an a.a. function on G_k. In addition we require that $\sup_k \|f_k\| < \infty$ where $\|\cdot\|$ is the supremum norm. A function $f(s)$ is defined on Z by

$$f(s) = f_k(s'), \; s = a_k + s', \; s' \epsilon G_k. \tag{3}$$

Properties (a) and (c) above guarantee that f is well defined on all of Z.

THEOREM 1. *The function $f(s)$ defined in (3) is a.a. on Z.*

Suppose now that G_k is generated by s_k for each k. Define $f_k(s)$ on G_k as $f_k(s) = \exp(2\pi i s/s_{k+1})$, $k = 1, 2, \cdots$. There will exist a seq. $\{p_k\}$ such that for each k

$$\left|\exp(2\pi i p_k s_k/s_{k+1}) - 1\right| > 1. \tag{4}$$

Letting $f(s)$ be defined by (3) we clearly have $f(s + p_k s_k) \to f(s)$ for all s. If f were a.p., this limit would already be uniform. However, for $s \epsilon A_k$ we have from (4) that $\left|f(s + p_k s_k) - f(s)\right| > 1$ for each k. Thus $f(s)$ is not a.p. and we have proved by example

THEOREM 2. *There exist a.a. functions on Z which are not a.p.*

If we fix a real number $y > 0$, an integer $r \geq 2$, and a bounded function $f(n)$ on Z, the function

$$\varphi_y(z) = \sum_{-\infty}^{\infty} \frac{f(n)}{(z + iy + n)^r} \tag{5}$$

will be uniformly bounded for complex numbers z with $Im(z) \geq 0$.

THEOREM 3. *The function $\varphi_y(z)$ of (5) is a.a. on each line $Im(z) = y_0 \geq 0$ if, and only if, $f(n)$ is a.a. on Z. Furthermore, $\varphi_y(z)$ is a.p. if, and only if, $f(n)$ is a.p.*

We introduce the notion of a Bohr a.a. function. Except for our boundedness condition these functions are the same as the N-a.p. functions of B. M. Levitan.[4]

Definition 2: A bounded function $f(s)$ on Z shall be called *Bohr a.a.* if for fixed $s \epsilon Z$ and $\epsilon > 0$ there exists a set $B_\epsilon = B_\epsilon(s)$ such that

(α) B_ϵ is relatively dense

(β) $B_\epsilon = -B_\epsilon$

(γ) $\tau \epsilon B_\epsilon \Longrightarrow |f(s + \tau) - f(s)| < \epsilon$

(δ) $\tau_1, \tau_2 \epsilon B_\epsilon \Longrightarrow |f(s + \tau_1 + \tau_2) - f(s)| < 2\epsilon$.

We now state two theorems upon which we shall elaborate at a later time. (Theorem 5 below has been stated and proved by B. M. Levitan for his N-a.p. functions.[4])

THEOREM 4. *A function $f(s)$ is Bochner a.a. if, and only if, it is Bohr a.a.*

Definition 3: A family $\{\varphi_\alpha\}$ of Bohr a.a. functions shall be called jointly a.a. if it is uniformly bounded, and if for fixed $s \epsilon Z$ and $\epsilon > 0$ there exists a set B_ϵ satisfying $\alpha) - \delta)$ for all but at most finitely many φ_α.

THEOREM 5. *A function $f(s)$ is Bochner a.a. if, and only if, it is the pointwise limit of a jointly a.a. sequence of trigonometric polynomials.*

The results announced here form part of the author's forthcoming doctoral thesis. I would like to express my thanks to Professor S. Bochner for suggesting the topic of almost automorphy and for many inspiring discussions on this and related topics.

* National Science Foundation Fellow.

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*COLANIC ACID**

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The strain of *Escherichia coli* which elaborates colicine K, and which this laboratory has studied for some time, has undergone mutation over the years. When this microorganism was first sent us by Dr. Pierre Fredericq in 1952, we observed that it grew on Neo-peptone agar as translucent colonies, some 3–4 mm in diameter, which were slightly rough. The bacillus was subsequently typed for us by Dr.

Fritz Ørskov of the Statens Seruminstitut, Copenhagen, and was found to have the serotype 01:K1. Not long thereafter a single opaque colony was observed growing on a plate which had been streaked with the translucent variant. This variant bacillus bred true on subculture, and because it yielded much more colicine than did the parent, it was chosen for our studies on the nature of colicine K.[1] Within the past several years this bacillus has again changed. Its colonial form is now larger (5–6 mm) and it grows as glistening, domed mucoid colonies which are also opaque. All three of these variants are colicinogenic, i.e., they elaborate an O antigen which is bactericidal and which we believe to be identical with colicine K. All three evoke antibodies in rabbits which agglutinate the parent cell, but only in low dilutions of antiserum (1:160). Because of this the microorganisms are said to contain a K (Kapsel) antigen which masks the underlying type specific O antigenic complex.[2a] Upon heating any one of the variants at 100° for 1–2 hr, the K antigen is destroyed, and the type specific O antigen is revealed; these same sera now agglutinate the heated microorganisms at very high dilutions (1:12,000).[2b] Because of the peculiar thermolability of the K antigen, it is subclassified as an "L" (labile) antigen, and henceforth it will be referred to as such.

The nature of the substance which is elaborated by the two opaque colonial forms of *E. coli* K235, and which we believe contributes to their mucoid characteristics, is the subject of this communication. At first we believed this material to be identical with the L antigen, but it will be seen that this is probably not the case. This mucoid substance, which we have named colanic acid, is a nitrogen-free, acidic polysaccharide. It is serologically active, and is endowed with a serological specificity which differs from that of the L antigen.

Materials and Methods.—Bacterial strains: Three strains of *Escherichia coli* were used in this study and were designated as *E. coli* K235 L + T, L + O, and L + O(m). The letter L signifies that each contains a thermolabile L antigen.[2a] The letter T indicates that the particular colonial form of the bacillus is translucent when grown on Neo-peptone agar. The designation O signifies that this colonial form is opaque. We have on hand two such variants: the colonies of the normal opaque form (*E. coli* K235 L + O) are the same size as those of the L + T variant. They are not particularly mucoid, whereas those of the m variant are larger, and are very mucoid indeed.

Antisera: Antisera to the three variants were prepared by injecting rabbits with acetone-killed bacilli,[3a] using a schedule of immunization suggested by Baker.[3b] Antisera to colanic acid itself was obtained by the intravenous injection of a sterile solution of pure polysaccharide in doses of 0.2, 0.3, 0.5, 1.0, and 2.0 mg administered at three-day intervals. In all instances the animals were exsanguinated five days after the last injection. Agglutination and precipitin tests were conducted as previously described.[4] The absorption of the various antisera with living microorganisms was performed in the conventional manner.[5] Absorption with colanic acid or with colicine K was performed by adding an accurately predetermined quantity of the precipitinogen to a measured sample of immune serum. After incubation at 0°C for 72 hr, the absorbed serum was centrifuged in the cold, and the supernate, containing a slight excess of the precipitinogen, but no specific antibody, was removed and used for the serological tests in question.

Toxicity tests: The toxicity of various preparations of colanic acid was determined by injecting Swiss mice with 4, 2, 1, and 0.5 mg quantities of the polysaccharide according to the technique of Schaedler and Dubos.[6]

Results.—Isolation of colanic acid: When *E. coli* K235 L + O(m) is grown under appropriate conditions[1] in a nutrient medium containing only dialyzable components, it elaborates sizable quantities of the type specific O antigen (colicine K),[7] colominic acid,[8] and the acidic polysaccharide colanic acid, together with some small amounts of other ill-defined nondialyzable substances of bacterial origin.

These products of bacterial synthesis can be isolated from the cell-free medium by freeze drying, after concentration *in vacuo* (4 L/hr, 18°C) and dialyzing free of all diffusible components (yield 7.5 gm). The material is now precipitated from solution (400 ml) at 0°C with ethanol (3 volumes), a procedure which eliminates both pigment and lipid. The precipitate is collected by centrifugation at 0°C, redissolved, dialyzed, and dried from the frozen state (yield 6.5 gm). A solution of the material (2 per cent) is then brought to 90 per cent saturation by the addition of solid ammonium sulfate. The O antigen and colanic acid precipitate, leaving the colominic acid in the supernatant. Three precipitations suffice to separate the major part of the colominic acid (2.2 gm) from the other two components. The ammonium sulfate precipitate containing colanic acid and colicine K, together with small amounts of colominic acid and nucleic acid, is now thoroughly dialyzed and dried from the frozen state (yield 3.8 gm).

This substance is next dissolved in 0.1 *M* phosphate buffer (300 ml, pH 7.2) and 75 ml of a solution of cetavlon is added.[9] The colanic acid precipitates and is thus separated from the major part of the O antigen. The substance is not pure, however, for it still contains a small amount of O antigen, and some colominic and nucleic acids. The cetavlon precipitate is dissolved in 200 ml of 1 *M* NaCl and the crude polysaccharide is precipitated with ethanol (3 vol). A substance is finally obtained, rich in colanic acid but still containing O antigen (15 per cent), colominic acid (20 per cent), and a small amount of nucleic acid. These substances are presumably bound by electrostatic forces, and conventional fractionation procedures fail to separate them. However, by repeatedly shaking a 1 per cent solution (0.05 *M* acetate buffer, pH 5.0) with chloroform-octyl alcohol (3:1),[10] followed by high speed centrifugation, it is possible to separate the O antigen. The latter remains in the chloroform emulsion and is discarded. The remaining colominic acid, together with small amounts of nucleic acid, can be separated by percolating a 1 per cent solution (1.4 gm in 140 ml of 0.05 *M* acetate buffer, pH 5.0) through a short column of DEAE-cellulose (3 gm packed in a column 1.8 × 10 cm). The filtrate contains the colanic acid free of colominic and nucleic acids; the latter remain on the DEAE-cellulose. After dialysis, the colanic acid is recovered by freeze drying (yield 0.7 gm from 15 liters of bacterial culture).

Both the normal opaque form of *E. coli* K235 and its mucoid variant yield colanic acid, though the latter yields nearly three times as much. The transparent variant, *E. coli* K235 L + T, does not elaborate any demonstrable colanic acid in the culture medium. This does not, however, preclude the possibility of small amounts of colanic acid being distributed on the cell surface. This question will be dealt with later.

Chemical properties of colanic acid: Colanic acid is obtained as a white, fibrous, amorphous substance which is soluble in water and in dilute salt solutions. It yields clear solutions which are highly viscous. The nitrogen content of various preparations varies between 0.1 and 0.3 per cent. The polysaccharide has a C and H content of 46.38 and 5.96 per cent, respectively. A 0.5 per cent solution of an electrodialyzed preparation of colanic acid has a pH of 2.5. The substance itself has an acid equivalent of 468. When the electrophoretic pattern[11] of colanic acid is examined (veronal buffer, pH 8.6), the substance appears homogeneous (Fig. 1). Because of the viscosity, good patterns are difficult to procure. The substance

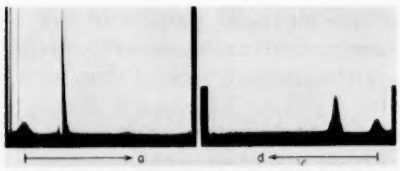

FIG. 1.—Electrophoretic pattern of colanic acid.

(*a*) Ascending boundary of a solution of colanic acid in 0.1 *M* veronal buffer at pH 8.6 (concentration 5 mg/ml) after 1860 seconds.

(*d*) Descending boundary. $\mu = -10.1 \times 10^{-5}$ cm^2 volt^{-1} sec^{-1}.

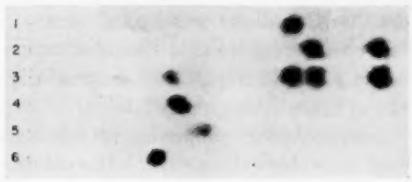

FIG. 2.—Chromatogram of sugars present in an acid hydrolysate of colanic acid. (1) Galactose (20γ). (2) Glucose, fucose (20γ each). (3) Hydrolysate of colanic acid (100γ). (4) Glucuronic acid (20γ). (5) Mannuronic acid (20γ). (6) Galacturonic acid (20γ). Chromatogram developed in pyridine, ethyl acetate, acetic acid, water (5:5:1:3) for 24 hr at 23°C, using the descending technique. Sprayed with $AgNO_3$ in acetone, then NaOH in ethanol. Fixed in sodium thiosulfate solution.

has a relatively high mobility ($\mu = -10.1 \times 10^{-5}$ cm^2 volt^{-1} sec^{-1}), indicating that the polysaccharide does indeed have strong acidic properties. Colanic acid gives a color reaction for uronic acids when tested with naphthoresorcinol or with Dische's carbazole reagent.[12] A quantitative estimation by the latter method reveals that the polysaccharide contains approximately 14 per cent of uronic acid calculated as glucuronic acid. A 1 per cent solution of colanic acid in 1 *N* H_2SO_4 is completely hydrolyzed at 100° within 160 min and yields some 80 per cent of reducing sugar, calculated as glucose. If such a solution is freed of sulfate ions, concentrated, and chromatographed on Whatman No. 1 paper, the sugars and the uronic acid which are present in the hydrolysate can be readily identified.[13] The polysaccharide is found to be constituted from glucose, galactose, fucose, and glucuronic acid (Fig. 2).

Toxicity of colanic acid: Certain preparations of colanic acid exhibit mild toxicity when injected intraperitoneally into mice. Although the polysaccharide does not cause death of the animals, nevertheless they suffer slight malaise and some diarrhea. The animals lose weight and fail to consume their normal complement of water within the first 48 hr. Their symptoms are typical of those exhibited by animals injected with very small quantities of endotoxin. Although one cannot demonstrate the presence of any O antigen in purified preparations of colanic acid by direct serological test in anti-colicine K immune serum, yet the sera of rabbits which have been injected with highly purified colanic acid will, in some instances, show the presence of very feeble precipitins for pure colicine K. Since the latter is an exceedingly efficient antigen, it seems that some of our preparations of colanic acid do indeed contain traces of O antigen, enough to render the polysaccharide slightly toxic and to evoke a feeble colicine K antibody response, yet insufficient to be

FIG. 3.—Gel precipitation reaction of colanic acid and colicine K in antibacterial serum before and after absorption. In each of the figures the upper left-hand well contains colanic acid, 2 mg/ml; the upper right-hand well contains colicine K, 2 mg/ml. In (*a*), the lower well contains *E. coli* K235 L + O(m) antiserum; (*b*) serum absorbed with colicine K; (*c*) serum absorbed with colanic acid; (*d*) serum absorbed with colicine K and with colanic acid.

detected by direct serological means. We make particular mention of this in order to emphasize that the purification of macromolecular substances of bacterial origin can be fraught with unusual difficulty and that the criteria of their purity can at times be singularly fallible.

Immunological properties of colanic acid: (a) *Serological specificity:* Colanic acid is serologically active. If a solution is titrated against an antibacterial serum elicited by the homologous microorganism *E. coli* K235 L + O (m), the polysaccharide gives a powerful precipitin reaction, which can still be detected in concentrations of 1 part in 6 million (Table 1). When tested against this same

TABLE 1

PRECIPITIN REACTION OF COLANIC ACID IN SERA OF RABBITS IMMUNIZED WITH *E. coli* K235 L + O(m)

Serum no.	Final dilution of test antigen 1:2,000	1:10,000	1:50,000	1:250,000	1:1,250,000	1:6,250,000
96	2	3	3	3	2	1/2
98	1/2	1/2	2	3	2	1
99	2	3	3	3	2	1/2
100	2	3	4	3	2	1/2

In Tables 1 and 2, 4 = heavy disk-like precipitate, clear supernate; 3 and 2 = partial precipitation; 1/2 = trace reaction.

serum by agar diffusion,[14] the carbohydrate shows but one serologically active component (Fig. 3, *a* and *b*, left well). That the substance is different serologically from the O antigen (i.e., colicine K) derived from the same microorganism is evident from the fact that the lines formed by an authentic sample of the latter and colanic acid cross (Fig. 3*a*). Furthermore, if such a serum is first absorbed with pure colicine K (Fig. 3*b*), it still reacts with colanic acid and vice versa (Fig. 3*c*). If the immune serum is absorbed with both, there is no reaction (Fig. 3*d*).

TABLE 2

PRECIPITIN REACTIONS OF COLANIC ACID AND COLICINE K IN SERA OF RABBITS IMMUNIZED WITH COLANIC ACID

Serum no.	Test antigen	Final dilution of test antigen 1:2,000	1:10,000	1:50,000	1:250,000	1:1,250,000	1:6,250,000
37	Colanic	1	0	0	0	0	0
38	acid	0	0	0	0	0	0
39		3	4	4	3	2	1/2
40		1	3	3	2	1	0
37	Colicine	1/2	1	2	1/2	0	
38	K	0	0	0	0	0	
39		1/2	1/2	1/2	1/2	0	
40		1/2	0	0	0	0	

(b) *Antigenicity of colanic acid:* Purified colanic acid will incite the formation of specific precipitating antibodies in experimental animals. Thus, two of four rabbits which were injected with purified colanic acid by the procedure outlined at the beginning of this communication yielded good precipitating antisera. That the antibodies are specifically directed against colanic acid itself and not the O antigen is evidenced by the fact that the latter, when tested in these same antisera, gives little or no precipitation (Table 2).

(c) *"Quellung" reaction of E. coli K235 L + O(m):* One of the typical serological characteristics of encapsulated microorganisms is their ability to exhibit "quellung" or swelling in homologous antiserum. This phenomenon, first described by Neufeld,[15]

is due to an interaction of the capsular polysaccharide with its specific antibody. We have observed that when the mucoid form of *E. coli* K235 is grown for short intervals (4–5 hr) in Todd-Hewitt broth,[16] it too exhibits this phenomenon. Furthermore, if these same bacilli are stained with India ink, they show the outlines of a capsule. For these reasons and for reasons set out below, it is believed that colanic acid encapsulates the cell wall of the mucoid microorganism. This does not appear to be the only immunologically active component present in the capsule, however, for reasons which will presently be seen.

Agglutination reactions of E. coli K235: Young cultures of *E. coli* K235 L + O(m) will agglutinate in low dilutions of an antiserum elicited by colanic acid, as may be seen in Table 3. If the colanic acid antibodies are removed by absorption with the

TABLE 3

AGGLUTINATION OF *E. coli* K235 L + O(m) AND L + T IN COLANIC ACID ANTISERUM

Serum no.	Strain of *E. coli* K235 tested	Final dilution of antiserum					
		1:10	1:20	1:40	1:80	1:160	1:320
39	L + O(m)	4	4	2	2	½	0
"	L + T	0	0	0	0	0	0
39A	L + O(m)	0	0	0	0	0	0

Serum 39 = Serum of rabbit immunized with colanic acid. Serum 39A = Serum 39 absorbed with colanic acid. In Tables 3 and 4, 4 = complete agglutination, clear supernate; 3 and 2 = partial agglutination; ½ = trace agglutination.

polysaccharide, the serum no longer agglutinates the bacillus. Here indeed is proof that agglutination of this microorganism takes place by virtue of the interaction of its encapsulating polysaccharide with homologous antibody. It is of interest that the L + T variant does not agglutinate in the unabsorbed immune serum, a fact which substantiates our observation that this microorganism does not synthesize colanic acid.

The agglutination reactions of these same two microorganisms in an antibacterial serum are not so simple, however. Both agglutinate in an antiserum to the mucoid bacillus (Table 4). The L + T variant reacts by virtue of its surface L antigen,

TABLE 4

AGGLUTINATION OF *E. coli* K235 L + O(m) AND L + T IN ANTIBACTERIAL SERUM BEFORE AND AFTER ABSORPTION

Serum no.	Strain of *E. coli* K235 tested	Final dilution of antiserum					
		1:10	1:20	1:40	1:80	1:160	1:320
100	L + O(m)	4	4	4	3	2	0
	L + T	4	4	3	1	0	0
100A	L + O(m)	4	4	3	1	0	0
	L + T	4	4	2	1	0	0
100B	L + O(m)	—	3	3	2	½	0
	L + T	—	0	0	0	0	0
100C	L + O(m)	—	0	0	0	0	0
	L + T	—	0	0	0	0	0

Serum 100 = Serum of rabbit immunized with *E. coli* K235 L + O(m). Serum 100A = Serum 100 absorbed with colanic acid. Serum 100B = Serum 100 absorbed with *E. coli* K235 L + T. Serum 100C = Serum 100 absorbed with *E. coli* K235 L + T and with colanic acid.

whereas the mucoid variant does so by virtue of both its L antigen and the encapsulating colanic acid antigen. If such a serum is absorbed with colanic acid, it still agglutinates both bacilli, though the titer for the mucoid variant is diminished, as one would expect. On the other hand, the titer for the L + T variant does not diminish—a fact which indicates that L antibody has not been removed.

If the antibacterial serum is now deprived of its L agglutinins by absorption with the L + T bacillus, the mucoid variant still agglutinates because the encapsulating colanic acid still reacts with its specific immune body. Upon absorbing this same immune serum still further with purified colanic acid, it will be seen from the table that the residual agglutinins for the mucoid variant are removed.

There is, of course, one other consideration which has not been taken into account in this analysis: both variants share a common O antigen (colicine K). However, this constituent does not participate in the agglutination reactions of young living *E. coli* which elaborate K antigens, a fact which has frequently been demonstrated.[2a]

Although all aspects of the serological analysis of these two organisms have not been presented, those which have are more than adequate to establish the fact that *E. coli* K235 L + O(m) elaborates two surface antigens, both of which participate in the agglutination of the cell in an homologous antiserum. One is the acidic polysaccharide, colanic acid; the other is its L antigen, a substance of unknown nature. *E. coli* K235 L + T, on the other hand, possesses but one surface antigen, an L antigen which appears to be identical with that of the mucoid variant.

Discussion.—The isolation of encapsulated mucoid strains of colon bacilli from the ileum of calves suffering from scours was reported by Theobold Smith in the late twenties.[17] In three masterful communications he described the colonial characteristics of these organisms and their variants, and related them to their pathogenicity and serological properties. It remained for his daughter, Dorothea, to isolate from one of these mucoid variants a polysaccharide which proved to be serologically active.[18] There are frequent references in the older literature to a so-called "slime wall" substance elaborated by other species of *Enterobacteriaceae*.[19] Perhaps the best studied of these is the mucoid substance so frequently encountered in strains of *S. paratyphoid* B.[20] This was shown to be a nitrogen-free polysaccharide[21] and is apparently elaborated by a variety of different Salmonella.[22] More recently Henriksen studied the serological properties of some thirteen mucoid strains of *E. coli* isolated from persons suffering from infections of the urinary tract. He has shown that these mucoid strains possess a common capsular antigen which he has called the M antigen. This substance is presumably a polysaccharide and bears a very close relationship to the slime wall antigen of *S. paratyphoid* B, if, indeed, it is not identical with it.[23]

In the present communication we have described the isolation and properties of a polysaccharide, which we have named colanic acid, from a colicinogenic strain of *E. coli* designated as K235. This substance has been obtained in a state of considerable purity and we have presented evidence which indicates that it encapsulates the organism and endows it with its mucoid characteristics. It has been shown, furthermore, that colanic acid is serologically active and functions as an excellent antigen in its own right. This is a fact of some note for, in the experience of the writer, which has not been inconsiderable, only one other native polysaccharide of bacterial origin has been encountered which is antigenic in rabbits.[24]

At first we thought colanic acid to be identical with the so-called L antigen of the bacillus. This is probably not the case, however, for *E. coli* K235 L + T neither agglutinates in an anti-colanic acid immune serum, nor does it elaborate any demonstrable quantity of colanic acid. We are unable to say at the present time whether colanic acid itself is identical with the nitrogen-free polysaccharide isolated

by Dorothea Smith, or with that obtained from *S. paratyphoid* B.[21] It is, however, quite different from the Vi polysaccharides isolated by Baker[25] from cultures of *S. typhosa*, *S. ballerup*, and a strain of *E. coli*. Dr. Baker kindly supplied us with a sample of his polysaccharide and with immune sera as well, but we were unable to find any serological crossing with his carbohydrate and ours.

In conclusion, let it be said that we believe colanic acid not only encapsulates the two opaque variants of *E. coli* K235 and masks the O antigen of these bacilli, but that it participates in their agglutination reactions. Both of these variants, and the L + T variant as well, share an additional surface antigen, a thermolabile L antigen which also contributes to the immunological characteristics of the cell. The nature of this substance, however, remains unknown.

Summary.—The colicinogenic bacillus *E. coli* K235 L + 0 (m) elaborates a serologically active, nitrogen-free polysaccharide which we have named colanic acid. The isolation of this substance and its immunological properties have been described. Colanic acid is a capsular polysaccharide which confers upon the bacillus its mucoid characteristics and certain of its serological properties as well.

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THE INFRARED SPECTRAL CHARACTERIZATION OF COLANIC ACID

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The isolation and properties of colanic acid, a serologically active polysaccharide which is elaborated by the colicine K-producing strain of *E. coli* K235, was described in a preceding communication.[1] During the course of this study a procedure was devised which proved satisfactory in eliminating small amounts of certain tenaciously held contaminants and thus has provided essentially pure colanic acid for characterization. One of the analytical procedures which was used in following the course of purification was the determination of the infrared spectra of the various preparations. This paper briefly reports the final spectral characterization of purified colanic acid.

It will be recalled that, in addition to colanic acid and the type specific O antigen, this bacillus also elaborates colominic acid, a polymer of N-acetyl neuraminic acid.[2] It is this substance, together with small quantities of O antigen, which proved most difficult to separate from colanic acid. Two preparations of the polysaccharide were finally obtained, Nos. 13 and 14, which were essentially free of these substances according to chemical and serological assay; both were made by the same procedure, except that preparation No. 13 had been further subjected to electrodialysis and hence was ash free and in the acidic form. The nitrogen content of these two preparations was 0.23 and 0.33 per cent, respectively, and was presumed to be due to small amounts of residual nondialyzable nitrogenous material.

The infrared characterization of the two preparations was made on lyophilized samples dispersed in KBr and pressed into 0.7 mm × 13 mm dia. pellets at a concentration of 2 mg per cent (w/w). The spectra were determined on a Model 221 (Perkin-Elmer) spectrophotometer equipped with a NaCl-prism/grating interchange under optimal conditions of resolution for definitive comparison of the two purified materials. As a further quantitative characterization, empirical extinction values were determined for the two characteristic bands at 1615 cm^{-1} and 1730 cm^{-1} over the concentration range from 1 to 6 mg per cent (cf. Fig. 1).

The spectra of both samples, as shown in Figure 1, are typical of polysaccharides containing a uronic acid.[3] Due to the electrodialysis, the essential difference between the two preparations lies in the conversion of the salt to the free acid form. This difference is revealed by a relative fall of the COO^- absorption bands at 1387 cm^{-1} and 1615 cm^{-1} in the spectrum of the electrodialyzed preparation (No. 13) with respect to that of the undialyzed substance (No. 14), and a rise of the COOH band at 1730 cm^{-1}. It appears, however, that this absorption change is superimposed upon an underlying absorption pattern consisting of two bands at 1630 cm^{-1} and *ca.* 1730 cm^{-1}, since these two bands persist in both the salt and acid forms, and since the band at 1630 cm^{-1} is definitely shifted with respect to the COO^- absorption at 1615 cm^{-1}. No definite assignments can be given to these two bands, but they could correspond to an amino and a noncarboxylic carbonyl absorption, respectively. There is an indication of the loss of a shoulder absorption (NH) on the main hydroxylic band at 3500 cm^{-1} which appears in the spectrum of

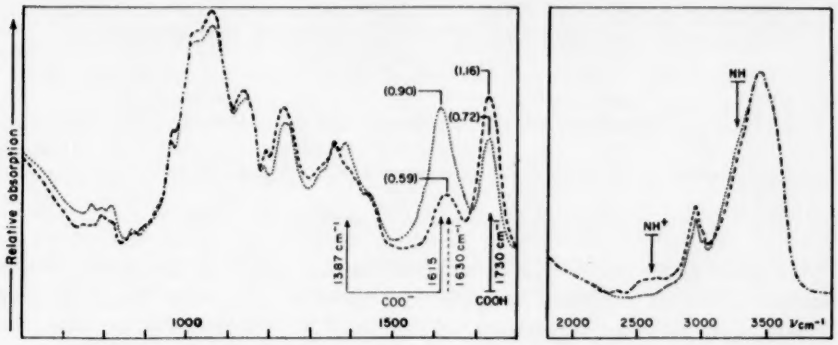

FIG. 1.—The infrared spectra of undialyzed (Prep. No. 14, ····) and electrodialyzed (Prep. No. 13, ----) colanic acid in potassium bromide at a concentration of 2 mg per cent by weight. The band assignments and positions referred to in the text are as indicated, with the corresponding empirical extinction values, E_ν (1 mg %, 0.7 mm) shown in parentheses.

the electrodialyzed preparation as an NH^+ band at *ca.* 2500 cm^{-1}, thus definitely confirming the presence of an amino group in both preparations. The lack of an amidic absorption band at 1540 cm^{-1} in the spectrum of colanic acid, which is a strong characteristic band in the spectrum of colominic acid[3] and that of peptides, indicates that there is little, if any, contamination due to these particular substances. A further spectral difference which might be noted occurs at about 800 cm^{-1} in the region associated with skeletal ring vibrational modes, and in all likelihood it is related to a change in the charge of a ring substituent group effected by acidification or neutralization, such as would be expected for the carboxyl group in these acid polysaccharides.

In conclusion, it may be stated that a limiting and characteristic infrared spectrum has been obtained for the purified salt and acid forms of colanic acid. The spectra reveal the presence of an amino functional group which might well be a constituent part of the intrinsic structure of this acidic polysaccharide, but which more probably is associated with a strongly adsorbed basic contaminant.

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CELLULAR ALTERATIONS IN THE MCH LINE OF CHINESE HAMSTER CELLS FOLLOWING INFECTION WITH HERPES SIMPLEX VIRUS*

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We previously reported the isolation of the aneuploid MCH line of Chinese hamster cells from a normal line originally derived by Dr. George Yerganian from female adult fibroblast derivatives which formed a short-term carrier state with herpes simplex virus.[1] We also reported the occurrence of chromosomal aberrations during the first postinfection division with this virus, and subsequently the presence of persistent chromosomal aberrations in clonal sublines derived from infected cells.[2] This paper is concerned with further studies of the effects of viral infection upon chromosomes of MCH cells.

Materials and Methods.—Cell line: The MCH line and the composition of the medium employed were previously described.[1] All sera used were heated to 56° for 40 min before use.

Virus: The SAE strain of herpes simplex virus used was previously described.[1] Virus was harvested from cultures of HeLa cells inoculated 24 hr earlier with 10^8 $TCID_{50}$ of virus. The cells were collected, washed 5–6 times with Hanks' balanced salt solution, and resuspended in 0.2 *M* PO_4 buffer containing 0.1% bovine serum albumin (Armour fraction V) at pH 7.0. The cells were then disrupted by 3 cycles of rapid freeze-thawing in a dry ice-alcohol mixture, followed by 4 min of sonication at maximum output in a 10 kc Raytheon sonic oscillator. Following disruption of cells, the suspension was centrifuged at 4°, the supernatant collected, and then filtered through a millipore filter (MF type HA) in a Swinny hypodermic adaptor before use.

Virus titrations were performed in six HeLa tubes per dilution, and the $TCID_{50}$ calculated by the method of Reed and Muench.[3] This strain of herpes simplex virus causes mainly a nonproliferative type of cytopathic effect in HeLa cells as described by Gray *et al.*[4]

Infection of cells: A stock bottle of MCH cells was trypsinized (0.25% Bactotrypsin-Difco) and centrifuged at room temperature. The resultant pellet was resuspended in medium, and the number of cells determined by direct count in a hemocytometer chamber. Three cc of a cell suspension containing 2×10^5 cells plus 0.1–0.2 ml of virus suspension or buffer was added to a 25 ml sterile plastic flask (Falcon Plastics). The cell-virus mixture was then placed on a rotating shaker and shaken (370 rpm) for 1 hr at 37°C. The suspension was either diluted directly for seeding of plates or first centrifuged and the cells washed to remove the bulk of residual virus. For cloning and plating efficiency studies, approximately 500 cells were seeded in Petri dishes. Within 24 hr the location of single attached cells was noted. These cells were observed during the next 7 days, and individual clones which were well isolated were removed with a capillary pipette and seeded in separate 25 ml plastic flasks. The flasks were incubated at 37° in an atmosphere of 5% CO_2 in air and refed every 2–3 days until a full cell sheet had formed. The cells were then trypsinized and passaged normally.

Virus antiserum: Antiserum was prepared in rabbits by intravenous inoculation of 10^8 $TCID_{50}$ of virus for six consecutive weeks. Neutralizing antibody was assayed in HeLa cells by a constant virus-varying serum dilution method following incubation at room temperature for one-half hour. Serum from a single animal having a titer of 1:130 for 10^5 $TCID_{50}$ virus was used throughout. Preinoculation bleeding from the same animal was used as control. All sera were heated to 56° for 40 min before use.

Chromosome preparations: Approximately 2×10^5 cells in 3 ml medium were seeded in glass Petri dishes containing 2 coverslips. After 48–72 hr, colchicine was added to yield a final concentration of 0.5 μg/ml. Plates were reincubated for an additional 2–4 hr. The medium was made hypotonic by adding ½ volume of prewarmed deionized water, and the plates were incubated

for 7 min. Cells were fixed in Carnoy's 3:1 at room temperature for 2–4 hr. The coverslips were stained with aceto-carmine and mounted on slides using a squash technique.

Cells were studied under the phase microscope at 250× magnification, and the location of intact cells with well-dispersed chromosomes was noted. These same cells were then studied under the oil immersion lens. Any cell whose chromosomal morphology was doubtful was photographed and an enlarged 8 × 10 print was made to facilitate study.

Results.—The MCH line has a consistent plating efficiency of 35–45 per cent. Even at viral multiplicities greater than 10^4 per cell, no decrease in plating efficiency was noted when virus was added either at the time cultures were initiated or subsequent to cell attachment. However, when the virus-cell suspension was shaken for 1 hr at 37°, cell killing as measured by a decrease in plating efficiency was observed. The linearity of the relation between cell survival and the amount of virus added (as shown in Fig. 1) indicates single-hit kinetics. Shaking by itself caused no decrease in the plating efficiency of control cells.

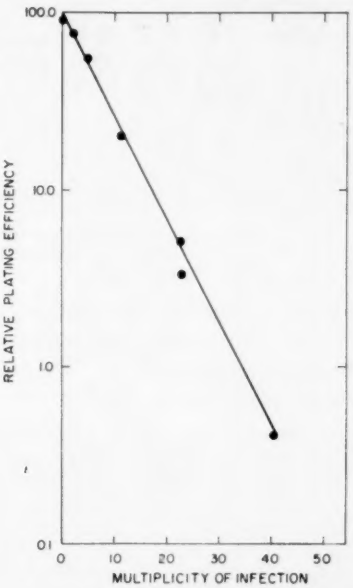

FIG. 1.—Cell survival following shaking of MCH cells with herpes simplex virus at various multiplicities.

Virus neutralized with specific antiserum or inactivated at 56° for 30 min did not cause a decrease in plating efficiency. Neither normal rabbit serum nor antiserum to virus had any effect on the plating efficiency of uninfected cells. Normal rabbit serum did not affect virus killing. Inoculation of control cells with phosphate buffer containing bovine serum albumin or extracts from uninfected HeLa cells had no effect. No cell killing was evident when cells were shaken alone and then virus added. The role of shaking in the production of the chromosomal abnormalities to be described is not understood.

The chromosomes of the MCH line are similar to those reported in other Chinese hamster lines with two exceptions. The first is the appearance of a distinctive marker (M) chromosome, apparently unique to the MCH line, and the second is a modification in the X_2-chromosome seen in over 70 per cent of the cells, where a deletion has occurred in the short arm. This latter alteration has been seen in other Chinese hamster lines.[5] The origin of the M-chromosome is not known, as the MCH line appeared spontaneously in a bottle seeded with a normal aneuploid (24–26 chromosomes) line of adult female Chinese hamster cells. The stemline number in the MCH line has remained at the 24 chromosome level with no tendency toward heteroploidy. There is, however, no regularity in the karyotype of these cells as regards the number of each chromosome type present. More than one M-chromosome per cell is rarely encountered. No variations in the number of sex chromosomes have been observed. We have never seen an uninfected MCH or clonal subline cell which did not contain a marker (M) chromosome.

Figure 2 shows comparative idiograms of an MCH cell and a diploid Chinese

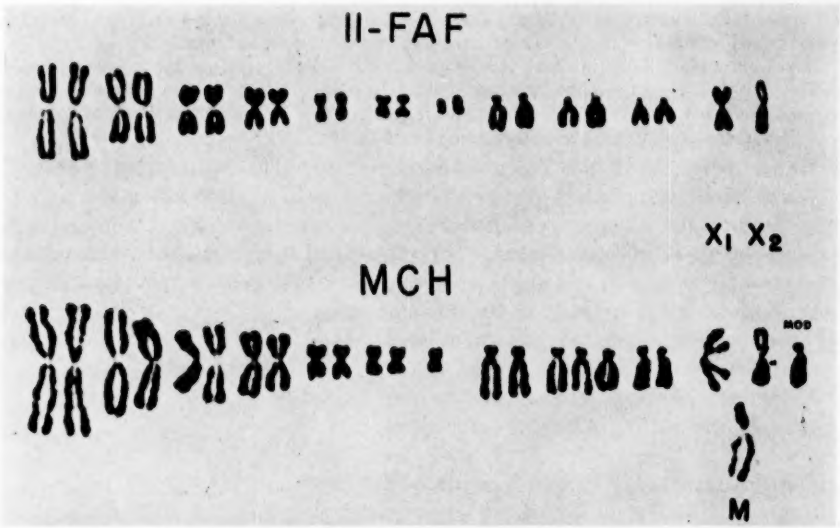

FIG. 2.—Idiograms comparing chromosomes of a normal diploid Chinese hamster cell (11-FAF) and an MCH cell. Both a normal and a modified X_2 are included for comparison. The marker (M) is seen only in the MCH line.

hamster cell from a line kindly supplied by Dr. Yerganian.[6] Except for the M and modified X_2, the chromosome arm ratios are very similar to those seen in other Chinese hamster cells.[7-9] The M-chromosome has an interarm ratio of 1.84 and the modified X_2, 4.0.

FIG. 3.—Chromosomes of a nonsurviving infected cell during the first postinfection division after shaking.

The chromosomes of infected shaken cells were studied during the period of their first cell division. Depending on the multiplicity of infection, severe chromosomal aberrations manifested by multiple breakages occurred during the first 3 days after infection of cultures as shown in Figure 3. The incidence of the aberrations paralleled the rate of cellular killing as determined by the decrease in plating efficiency. The aberration rate returned to the control level of 1–3 per cent by the fifth postinfection day, and remained so during subsequent passages. When the cells of later passages were studied with respect to possible chromosomal alterations, no differences from normal were evident. Strikingly different results were obtained when infected shaken cells were plated at low concentration immediately after infection as for plating efficiency studies, and individual clones isolated and propagated separately after 7 days.

TABLE 1

CHROMOSOME DELETIONS IN INFECTED AND UNINFECTED CLONES*

		Percent of Total Deletions — Chromosomes No.				
	No. deletions/cell	1	2	3	4	X_1
Control	0.025† (0.02–0.03)‡	25	25	47	3	0
Infected	1.9 (1–2.5)	16	52	22	5	5

* Represents data from 50 cells per clone.
† Average from all clones.
‡ Range among clones.

We have thus far studied 12 such infected and 17 control clones in detail. No degenerative changes appeared in any of the infected clones during the subsequent two-month period, nor were there any changes from normal in plating efficiency.

Chromosomes of all infected clonal lines studied 5–6 weeks after infection showed a number of alterations, many of which could be identified as surviving chromosome or chromatin deletions of specific segments on chromosomes Nos. 1, 2, and 3, following loss of the acentric fragments. The aberration rate in these cells varied from 1–3 per cent. Table 1 shows the number and involved chromosomes with stable changes having the major deletions that were found among infected and control clones. These do not include asymmetrical aberrations such as chromatid lesions or gaps, dicentrics, rings, and translocations. Deletions occurred at a frequency of 80–100 per cent in all infected clones, while in controls the maximum frequency was 3 per cent. Figure 4 shows the frequency of the major deletions at the various loci on chromosomes Nos. 1, 2, and 3. The numbers below chromosomes Nos. 1 and 2 represent the loci designated by Somers and Hsu[10] as involved in aberrations following treatment of Chinese hamster cells with 5-bromodeoxyuridine, hydroxylamine and X irradiation. We have seen no deletions on these chromosomes occur-

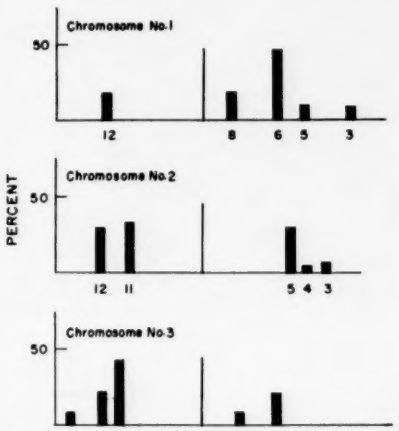

FIG. 4.—Diagrammatic representation of chromosomes Nos. 1, 2, and 3 showing the relative frequency of deletions at various loci in infected cells. The numbers below chromosomes Nos. 1 and 2 are taken from Somers and Hsu.[10]

TABLE 2

ANALYSIS OF 20 CELLS FROM TWO INFECTED CLONES SHOWING ALTERED CHROMOSOMES

	Cell No.	Chromosome affected		Cell No.	Chromosome affected	
Clone 4	1	2	Clone 8	1	2,3	
	2	2,3		2	2	X_1
	3	2		3	2	X_1
	4	1,2		4	2	
	5	2		5	3	
	6	2 4		6	2 4	
	7	1		7	2 4	
	8	2		8	2,3	
	9	2		9	2,3	X_1
	10	2		10	1,2,3	

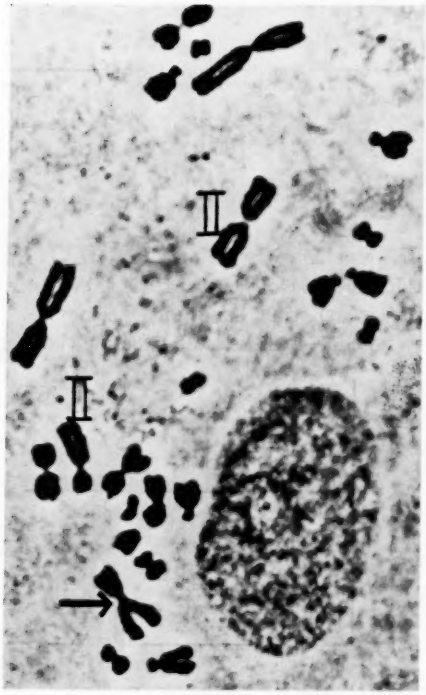

FIG. 5.—Chromosomes of an infected MCH cell showing one unidentifiable chromosome (arrow). One chromosome No. 2 has a deletion at position 11. No marker chromosome is evident.

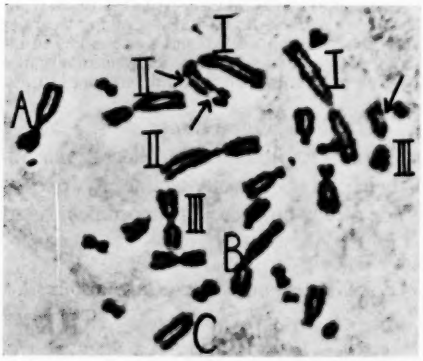

FIG. 6.—Chromosomes of an infected MCH cell. One chromosome No. 1 shows two chromatid breaks (arrows). One chromosome No. 3 shows a chromatid break (arrow). The chromosome labeled (B) is probably the X_1 with an elongated long arm. Chromosomes labeled (A) and (C) are unidentified. No marker chromosome is evident. One chromosome No. 2 has a deletion at position 11.

ring at loci differing morphologically from those reported by these authors. Deletions in control cells occurred at the same loci on chromosomes Nos. 1, 2, and 3.

While the relative distribution of affected chromosomes within each clone generally followed the scheme described in Table 1, there was always some variation within cells of a given clone as to which chromosomes were involved. Table 2 shows the analysis of 20 cells selected at random from 2 infected clonal sublines. Frequently a cell showed deletions in more than one chromosome. Rarely were both chromosomes of a pair involved within a single cell. These changes are apparently compatible with cell survival, as shown by the maintenance of normal plating efficiency. Deletions in control cells occurred at the same loci on chromosomes Nos. 1, 2, and 3.

Chromosome alterations other than deletions were also evident. In at least 3 of the infected clones, over 50 per cent of the cells showed no recognizable marker chromosome (Figs. 5 and 6). In two infected clones, almost every cell had at least one new chromosome which would not be identified, and was morphologically different from cell to cell (Figs. 5 and 6). Changes were also seen in the sex chromosomes; however, due to the similarity of the X-chromosomes to autosomes, we have not been able to make a systematic study of the frequency or morphology of these changes. Those changes which could be identified occurred mainly in the X_1-chromosome (Figs. 6–8). Some of these changes were reported to occur in both spontaneous and polyoma-induced tumor cells of Chinese hamsters.[7]

Discussion. — The MCH system shows arrested mitosis when killing

occurs following shaking of cells with virus. Killing is manifested by multiple chromosomal aberrations in colchicine blocked metaphase cells. The severity of the aberrations indicates that cell death results from the cells' inability to complete division. Apparently, this represents a special type of cytopathic effect for this virus. Stoker and Newton[11] and Roizman[12] reported arrested mitosis in herpes-infected cells, but no chromosomal aberrations were noted. Cell killing concomitant with multiple aberrations was seen in the first generation after X irradiation of Chinese hamster cells by Greenblatt.[13]

The reason for the apparent absence of permanent chromosomal alterations when infected shaken cells are seeded in large numbers (2×10^6), in contrast to cells cloned after infection, is not clear at the moment. It is possible that cell density influences the appearance of the alterations, and that the alterations are produced in cell lineages which have a selective disadvantage in mass cultures. The presence of alterations at high frequency in all infected clones is especially interesting owing to two characteristics of the process. The first characteristic is the different distribution of breaks in the cells of the same clone. This difference shows that breaks were formed in different cells during the growth of the clone. The observations would suggest that new breaks appear for several cell generations after infection. Thus, an agent causing breaks may persist in the cells for some time. The other characteristic is the location of the breaks. They are located at loci morphologically undistinguishable from those at which breaks occur following exposure of the cells to 5-bromodeoxyuridine, hydroxylamine, and X-irradiation treatment. This suggests that the location of the breaks is a property of the cell and not of the inducing agent.

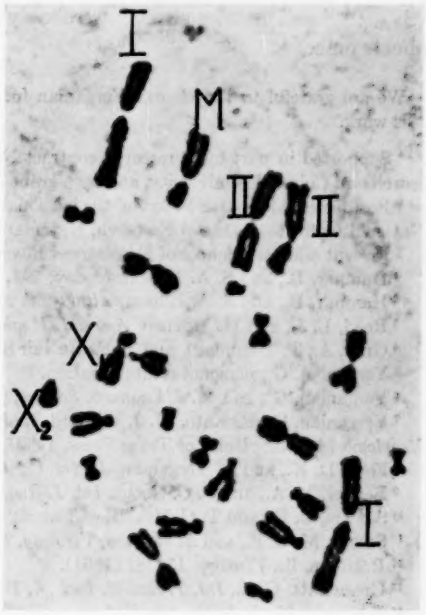

FIG. 7. Chromosomes of an infected MCH cell. One chromosome No. 2 has a deletion at position 11. The X_1-chromosome has a deletion in its short arm. The X_2-chromosome has a centromeric break with only one arm evident.

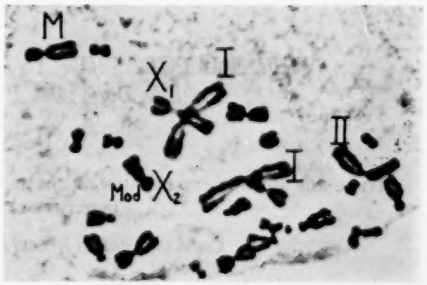

FIG. 8.—Chromosomes of an infected MCH cell. The X_1-chromosome shows an elongation of its long arm.

Subsequent to our initial findings, a number of reports have appeared of chromosomal involvement following infection of cells with simian vacuolating virus

(SV_{40})[14–16] and measles virus.[17] Probably other viruses will be found to have similar effects on cells.

We are grateful to Dr. George Yerganian for his assistance and advice during the course of this work.

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*THE DENATURATION AND THE RENATURATION OF THE DNA OF POLYOMA VIRUS**

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Polyoma virus induces neoplastic growths in some rodents and causes cytopathic effects in mouse embryo tissue cultures.[1] It contains DNA corresponding to a molecular weight of 7.5×10^6 per intact virus particle.[2] The genetic information for the replication of the virus and for the initiation of tumors in hamsters is contained in a subviral infective agent (SIA) which can be extracted from the virus[3, 4] with phenol. The SIA consists essentially of the viral DNA which is mainly base-paired.[5] From the buoyant density (1.709 gm cm^{-3}) in CsCl gradients and from the melting profile it can be estimated that the DNA of polyoma virus should contain about 49 mol % of guanine-cytosine. The presence of small numbers of unusual nucleotides or of small amounts of constituents other than DNA could not be excluded. Sedimentation velocity studies of phenol extracts from purified

polyoma virus by the newly developed band centrifugation[5a] showed the presence of three discrete fractions.

It will be shown in this paper that the DNA of polyoma virus exhibits some properties which have not been reported for other base-paired, functional DNA.

Material and Methods.—The SIA and the DNA from purified preparations of polyoma virus[6] were extracted with phenol in the presence of Na-trichloroacetate (pH 8.0) and EDTA according to a technique described previously.[4] The infective titer of phenol extracts was determined by a modified plaque assay method.[4]

The treatment of the phenol extracts with enzymes and with formaldehyde, and the methods used for the preparative and analytical sedimentation equilibrium centrifugation in CsCl density gradients, will be described elsewhere.[5] DNA from bacteriophage λ (b_2b_5c) was extracted with phenol and assayed for infectivity according to a method described by Kaiser.[9]

Thermal denaturation and thermal reannealing: Thermal denaturation and reannealing were performed essentially according to Marmur and Lane[7] and Marmur and Doty.[8] The buffers in which the extracts were heated at 100°C were either 0.1 *M* NaCl-0.05 *M* tris pH 8.0. 0.1 *M* NaCl-0.02 *M* citrate-phosphate, or 0.02 or 0.001 *M* citrate-phosphate, pH 7.0. The rapid cooling was done either by immersing the glass vials into ice water or by diluting the heated samples at the end of the heating period with 10 to 20 volumes of an ice-cold buffer. Thermal reannealing was done by adjusting the heat-denatured phenol extracts to 0.3–0.4 *M* NaCl (pH 7.0), followed by heating for 15 to 30 min at 70°C.

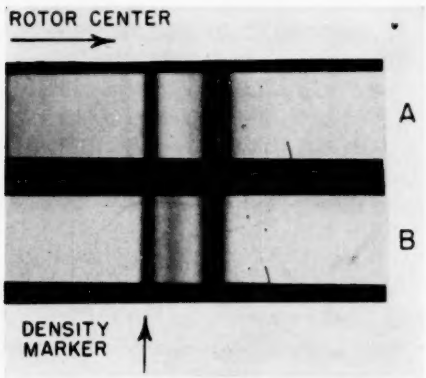

FIG. 1.—The behavior during sedimentation equilibrium centrifugation in CsCl of polyoma DNA freshly extracted from purified virus (DNA from *M. lysodeikticus*[13] used as a density marker, $\rho = 1.732$ gm cm^{-3}). (*A*) Native polyoma DNA. (*B*) Polyoma DNA (3γ/ml) heated for 5 min at 98.7°C in 0.01 *M* citrate-phosphate buffer, pH 7.0; quenched in ice water.

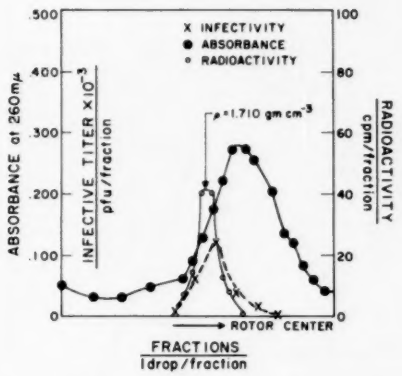

FIG. 2.—*Native polyoma SIA:* Untreated phenol extract from infected mouse embryo cell cultures. *E. coli* C^{14}-DNA added as a density marker ($\rho = 1.710$ gm cm^{-3}). Centrifuged in CsCl gradient at 35,000 rpm for 43 hr. Pfu = plaque-forming units.

Denaturation and reannealing by formamide: For the denaturation of DNA the procedures of Helmkamp and Ts'o,[10] and Marmur and Ts'o[11] were followed. Formamide (99%, Matheson, Coleman, and Bell) of low conductivity and neutral pH were used at concentrations ranging from 85–99% v/v. In all the experiments the final concentration of NaCl was below 0.01 *M*. The results were essentially the same whether the incubation in formamide had been carried out at room temperature (23°C) or at 50°C. The formamide was removed by dialyzing the samples at 4°C against buffers containing 0.1 *M* NaCl. In some experiments the reannealing procedure developed by Studier[12] was used: the denatured DNA preparation was reannealed for 2 hr at room temperature in 50% v/v formamide, in the presence of 0.5 *M* NaCl (neutral pH) and thereafter the formamide dialyzed out.

Results.—The DNA extracted from purified preparations of polyoma virus formed in the CsCl gradient in the analytical ultracentrifuge a single, rather broad band with the peak at a density of 1.709 gm cm^{-3} (Fig. 1*A*). Phenol extracts from infected mouse embryo tissue cultures were centrifuged under comparable conditions in the preparative ultracentrifuge. The mouse DNA formed a broad band. The peak of the band containing the infectivity of native phenol extracts nearly coincided with that of the radioactive *E. coli* DNA (1.710 gm cm^{-3})[13] used as a density marker (Fig. 2).

The denaturation of polyoma DNA: (*a*) *Thermal denaturation:* Polyoma DNA has a relatively sharp melting profile with the midpoint, T_m, at about 89°C (0.15 *M* NaCl). The transition was complete within a few minutes at 95°C as judged by the optical absorbance at 260 mμ. After heating and rapid cooling under conditions sufficient to melt polyoma DNA, the infective titer of phenol extracts containing the SIA regularly increased by a factor of about three. The effect was independent of the amount of heterologous DNA present (at least up to 300 γ/ml) and of the ionic strength within the range tested. The same increase was observed when the SIA had been subjected to density gradient centrifugation or when it had been incubated with chymotrypsin prior to the heating. In contrast, the DNA of bacteriophage λ, heated under the same conditions, lost about 99 per cent of the original infectivity. Slow cooling as well as reannealing at 70°C resulted in a marked decrease of the infectivity of phenol extracts containing the SIA with respect to rapidly cooled controls.

Upon heating at 100°C, followed by rapid cooling, the infectivity of phenol extracts was remarkably stable up to about 15 min (Fig. 3).

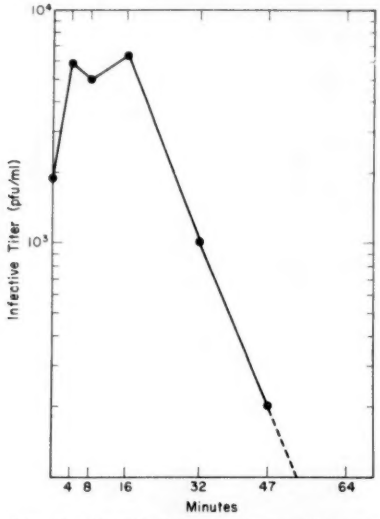

FIG. 3.—*Inactivation of polyoma SIA upon heating at 100°C:* Phenol extract heated at 100°C for the indicated lengths of time in 0.1 *M* NaCl-0.01 *M* tris pH 8.0. Aliquots cooled in ice water and immediately assayed for infectivity.

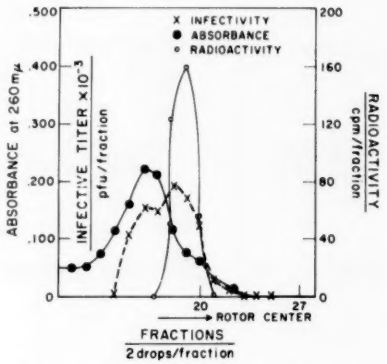

FIG. 4.—*Heated and rapidly cooled polyoma SIA:* Aliquot of the phenol extract shown in Fig. 1 heated at 100°C for 10 min in 0.1 *M* NaCl-0.05 *M* tris pH 8.0. Cooled in ice water. Native *E. coli* C^{14}-DNA added prior to density gradient centrifugation.

In the CsCl density gradient the infective material contained in phenol extracts which had been heated for 5 to 10 min at 100°C (followed by rapid cooling) formed two bands with about the same amounts of infectivity. The lighter band corresponded to the density of largely renatured, and the heavier band to the density of denatured, polyoma DNA (Fig. 4). The bimodal distribution was the same whether the heating at 100°C had been done in 0.1 *M* NaCl or in 0.001 *M* citrate-phosphate. It is likely that clusters made up entirely of the base-pair guanine-cytosine would have melted out in 0.001 *M* citrate-phosphate.[14] The assumption that the observed increase in the buoyant density of a fraction of the SIA was caused by the collapse of its secondary structure was further supported by the high sensitivity to formaldehyde[15] of the SIA in the band of higher buoyant density (Fig. 5). Native SIA showed a very low sensitivity to 1.8 per cent formaldehyde, (at 37°C, pH 7.0, about one "lethal hit" within 18 hours). With polyoma DNA freshly extracted from purified virus similar results were obtained upon heating at 100°C followed by quenching in ice water. The DNA formed two distinct bands in a CsCl gradient in the analytical ultracentrifuge. One band had the buoyant density of denatured, and the other that of largely renatured, polyoma DNA (Fig. 1*B*).

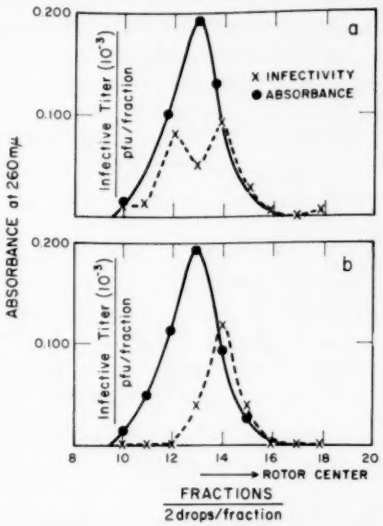

FIG. 5.—*Sensitivity to formaldehyde of heated and rapidly cooled polyoma SIA after CsCl density gradient centrifugation:* Phenol extract heated for 6 min at 100°C in 0.1 *M* NaCl-0.01 *M* tris pH 8.0, quenched in ice water, and centrifuged in a CsCl density gradient for 44 hr. Fractions collected and divided in two aliquots. Both aliquots incubated at 45°C for 40 min (pH 7.0); aliquot B in the presence of 1.8% formaldehyde. Samples dialyzed for 2 days prior to the assay for infectivity.

The DNA of bacteriophage λ was fully denatured after heating for 1 min at 100°C (followed by rapid cooling) as determined by the increase of the buoyant density in a CsCl gradient.

(*b*) *Denaturation by formamide:* Incubation with formamide under conditions which were adequate to denature and to inactivate preparations of λ DNA to about 1 per cent of the native control, did not result in a decrease of the infective titer of phenol extracts containing the SIA. Incubation in formamide in the presence of 1.8 per cent formaldehyde at neutral pH and room temperature resulted in a rapid and complete loss of the infectivity. In CsCl density gradients the banding pattern of the formamide-treated SIA was not significantly different from that of untreated controls (Fig. 6). The mouse DNA present in the treated phenol extracts, however, showed the expected increase in the buoyant density and the loss of the characteristic melting profile. It is likely that the SIA denatured during the treatment with formamide but that it renatured while the reagent was dialyzed out.

(*c*) *Denaturation under alkali and acid conditions:* Polyoma DNA denatured at 25°C around pH 12 and showed in CsCl density gradients the expected marked

shift toward a higher buoyant density.[16] Phenol extracts containing the SIA did not show any detectable loss of infectivity upon incubation at 37°C at pH 7.0 in 0.03 *M* citrate-phosphate buffer for at least 30 hr. Incubation at a pH of 3 or below resulted in a very markedly increased rate of inactivation. The relatively slow onset of the inactivation upon lowering the pH to 3 or below suggests that the inactivation was probably not due to the collapse of the helical structure *per se*.[17]

The renaturation of polyoma DNA: If phenol extracts were heated in 0.02 *M* citrate-phosphate at 100°C for 5 to 10 min and rapidly cooled, then incubated with 1.8 per cent formaldehyde, pH 7.0 at 37°C, about 95 per cent of the heated SIA was more than 200 times as sensitive to the reagent as the unheated SIA. About 5 per cent of the heated and rapidly cooled SIA showed a sensitivity to formaldehyde which was comparable to the low sensitivity of native SIA. The heated and rapidly cooled SIA remained equally sensitive to formaldehyde if it was stored in 0.02 *M* citrate-phosphate pH 7.0 for 24 hr at 4°C prior to the incubation with the reagent.

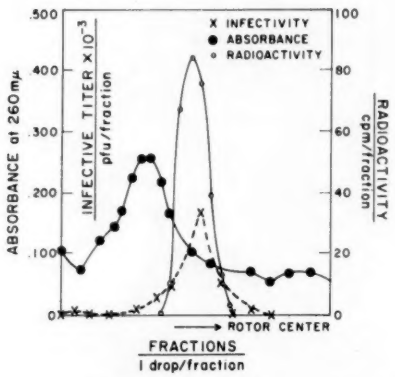

FIG. 6.—*Formamide-treated polyoma SIA:* Phenol extract incubated for 25 min at 37°C in 84% (v/v) formamide (0.01 *M* NaCl-0.005 *M* tris pH 8.0-0.001 *M* EDTA); thereafter dialyzed at 4°C against 0.1 *M* NaCl-0.05 *M* tris pH 8.0-0.001 *M* EDTA. Native *E. coli* C^{14}-DNA added prior to density gradient centrifugation.

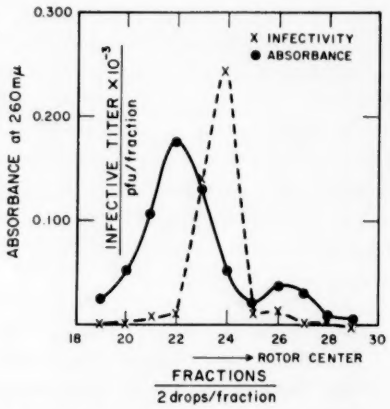

FIG. 7.—*Thermal reannealing of heated and rapidly cooled polyoma SIA:* Phenol extract heated at 100°C for 10 min in 0.1 *M* NaCl-0.01 *M* tris pH 8.0. Cooled in ice water. Reannealed for 30 min at 70°C in 0.32 *M* NaCl-0.03 *M* tris pH 8.0.

However, addition of 1.8 per cent formaldehyde immediately after the heating and cooling had little effect on the extent to which (purified) polyoma DNA renatured spontaneously, as determined by its buoyant density. Thus, it is probable that the reformation of the helical configuration took place during or soon after the cooling. The marked sensitivity to formaldehyde of most of the heated and rapidly cooled SIA, as detected by loss of infectivity, suggests that the arrangement of the complementary strands on most of the reformed helices was not fully regular immediately after the rapid cooling, and that a more regular arrangement took place in the CsCl solution (Figs. 4 and 5).

Preliminary experiments in the analytical ultracentrifuge showed that the extent to which purified polyoma DNA renatures spontaneously after heating and rapid cooling decreases as a function of the time of heating at elevated temperatures.

Similarly, "aged" (4°C) polyoma DNA showed a decreased tendency to renature spontaneously.

The renaturation of heat-denatured SIA occurred readily following incubation in formamide. Dialyzing the reagent out was sufficient for renaturation to occur. Similarly, thermal reannealing of heat-denatured SIA (Fig. 7) or (fully) heat-denatured purified polyoma DNA restored the buoyant density of the SIA and of the DNA nearly to that of the native controls. After thermal reannealing the SIA lost its high sensitivity to formaldehyde. Attempts to reanneal formamide-denatured preparations of λDNA (2 μg/ml) with formamide did result in a very small increase in the infectivity only.

Discussion.—The DNA of polyoma virus shows some properties which have not been reported for any other base-paired, functional DNA. Thus, the collapse of the secondary structure does not impair its biological activity. The differences of the effect of heating on polyoma DNA and transforming bacterial DNA can be explained, at least partially, by the properties of the assay systems used in the two cases.

The uptake of native as well as of denatured polyoma DNA by mouse embryo cells proceeds in at least two distinct steps:[18] a rapid initial attachment to the cells is followed, under adequate experimental conditions, by a time and temperature dependent process which possibly corresponds to the uptake of the DNA into the cells. Radioactive polyoma DNA which had been heated for 5 or for 20 min at 100°C (followed by rapid cooling) attached to mouse embryo cell monolayers 3 to 5 times more efficiently than the unheated DNA. Thus, the regularly observed increase in the infective titer of phenol extracts after heating under conditions where polyoma DNA melted out, at least temporarily, can possibly be explained by a more efficient initial attachment of the heated SIA to the mouse embryo cells. On the contrary, the uptake by intact bacterial cells of transforming DNA is sharply reduced upon denaturation.[19]

The stability of the infective titer upon heating at 100°C is not understood. The loss of infectivity upon reannealing for 30 min at 70°C stands in apparent contrast to the stability of the SIA when heated at 100°C (followed by rapid cooling). The reason for this is not known; it may be due partially to a reversal of the phenomenon leading to the increase in the infectivity after heating and rapid cooling.

The outstanding feature of the DNA of polyoma virus is its great readiness to renature. Because of its homogeneity, the renaturation of the DNA extracted from purified polyoma virus preparations is expected to occur readily under optimal reannealing conditions.[8] However, no other functional, helical DNA has been reported so far which shows a comparable tendency to renature spontaneously after heating and rapid cooling in buffers of relatively low ionic strength as does the DNA of polyoma virus. This behavior is somewhat reminiscent of that of helical DNA, the strands of which have been cross-linked by treatment with nitrous acid, bifunctional nitrogen mustard,[20] or by irradiation with ultraviolet light.[21] However, on the basis of the evidence presented, a complete separation of the complementary strands followed by a very rapid nucleation and reformation of the helical structure cannot be excluded.

The DNA's of two other mammalian viruses, adenovirus (type 2)[22] and pseudorabies virus,[23] with molecular weights similar to that of polyoma virus, show a

melting behavior which is in accordance with that reported for other base-paired DNA.

The biological significance of the above-mentioned properties of polyoma DNA is not known. The similarity in several respects of polyoma virus with at least two other tumor-producing viruses, the Shope papilloma virus[24] and the virus SV 40,[25] is remarkable. They are the only mammalian viruses so far reported to yield an "infective DNA" upon extraction with phenol.[26] The "infective DNA" of all three viruses shows a comparable thermal stability.[27, 28] The physico-chemical properties of the DNA of polyoma virus are similar in some respects to those of the DNA of Shope papilloma virus.[29] Preliminary experiments, however, suggest that the buoyant density of the DNA of the three viruses is not identical. A better understanding of the nature of the DNA of polyoma virus and that of related mammalian viruses has to be acquired in order to learn whether there might exist a causative relation between the observed properties of polyoma DNA and its oncogenic potential.

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AN APPROXIMATE CELL MODEL FOR LIQUID HYDROGEN, II

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In a previous publication[1] (hereafter called I), an approximate cell model was formulated and applied to liquid hydrogen. In this approximate model the potential well of Lennard-Jones and Devonshire[2] was approximated by a parabolic well split by a region of constant potential. This approximate cell model appears to preserve the essential features of the Lennard-Jones and Devonshire theory but does not involve the computational difficulties of the exact method of Levelt and Hurst.[3]

In this publication the variation of thermodynamic properties among the H_2 isotopes is reported.

Comparison of Tabulated Properties and Conclusions.—In Table 1 the reduced internal entropy is tabulated for the five H_2 isotopes. The reduced thermodynamic functions have been defined in I and the potential parameters ($\epsilon/k = 37.00°K$, $\sigma = 2.928$ Å) have been assumed to be the same for all five liquids. The appropriate values of the quantum parameters of these five liquids, obtained from Hirschfelder *et al.*,[4] have also been included in Table 1. The communal entropy correction has been introduced and, therefore, the internal entropy tends to zero at high temperatures and low densities. In Tables 2 and 3 the reduced internal energy and the reduced internal specific heat are tabulated. The internal energy and specific heat are in reasonable agreement with the calculations of Levelt and Hurst.[3] The agreement of the internal entropy with the calculations of Levelt and Hurst is less satisfactory.

As was reported in I, this approximate cell model does not give satisfactory values for volume derivatives. However, it does give reasonable results for the quantum correction. If the quantum correction to the reduced pressure is added to the reduced pressures, calculated by Hirschfelder *et al.*,[4] for the classical cell theory, a good estimate of the critical constants may be obtained. The calculated and experimental critical constants of the five H_2 isotopes are given in Table 4. The experimental critical constants for H_2, HD and D_2 were obtained by Hoge and Lassiter.[5] The "experimental" critical constants of DT and T_2 are given in parentheses, since they are estimates given by Friedman *et al.*[6] based on an assumed linear variation of the critical properties with λ^*. For comparison, the calculated and experimental critical constants for a classical liquid have also been included. The calculated

TABLE 1: REDUCED INTERNAL ENTROPY

T^*/v^*	1.00	1.20	1.40	1.60	1.80	2.00	2.25	2.50	2.75	3.00	3.50	4.00	5.00
						H_2 ($\lambda^* = 1.729$)							
0.40	−1.2392	−1.4142	−1.5320	−1.5762	−1.5488	−1.4707	−1.3370	−1.1930	−1.0547	−0.9281	−0.7118	−0.5375	−0.2754
0.50	−1.5707	−1.7239	−1.7854	−1.7505	−1.6474	−1.5120	−1.3338	−1.1666	−1.0170	−0.8851	−0.6662	−0.4927	−0.2343
0.60	−1.8335	−1.9469	−1.9382	−1.8309	−1.6738	−1.5051	−1.3057	−1.1293	−0.9763	−0.8436	−0.6259	−0.4545	−0.2002
0.70	−2.0425	−2.1021	−2.0233	−1.8590	−1.6671	−1.4801	−1.2708	−1.0914	−0.9379	−0.8059	−0.5904	−0.4215	−0.1710
0.80	−2.2065	−2.2055	−2.0647	−1.8588	−1.6451	−1.4484	−1.2350	−1.0550	−0.9024	−0.7715	−0.5588	−0.3921	−0.1453
0.90	−2.3330	−2.2710	−2.0789	−1.8438	−1.6168	−1.4151	−1.2004	−1.0202	−0.8699	−0.7405	−0.5304	−0.3660	−0.1225
1.00	−2.4281	−2.3089	−2.0761	−1.8208	−1.5860	−1.3820	−1.1676	−0.9896	−0.8399	−0.7121	−0.5045	−0.3422	−0.1019
1.10	−2.4981	−2.3272	−2.0627	−1.7937	−1.5545	−1.3500	−1.1366	−0.9602	−0.8121	−0.6858	−0.4808	−0.3204	−0.0830
1.20	−2.5474	−2.3316	−2.0430	−1.7648	−1.5234	−1.3192	−1.1073	−0.9327	−0.7863	−0.6614	−0.4587	−0.3003	−0.0655
1.30	−2.5804	−2.3263	−2.0193	−1.7353	−1.4930	−1.2898	−1.0797	−0.9069	−0.7620	−0.6385	−0.4381	−0.2814	−0.0492
						HD ($\lambda^* = 1.412$)							
0.70	−2.5586	−2.4560	−2.2128	−1.9390	−1.6883	−1.4721	−1.2463	−1.0601	−0.9041	−0.7713	−0.5564	−0.3889	−0.1414
0.80	−2.6741	−2.4921	−2.1993	−1.9052	−1.6480	−1.4310	−1.2068	−1.0227	−0.8689	−0.7380	−0.5264	−0.3613	−0.1176
0.90	−2.7502	−2.5010	−2.1738	−1.8680	−1.6085	−1.3924	−1.1704	−0.9887	−0.8370	−0.7080	−0.4993	−0.3367	−0.0962
1.00	−2.7968	−2.4930	−2.1424	−1.8204	−1.5709	−1.3564	−1.1369	−0.9576	−0.8079	−0.6807	−0.4748	−0.3143	−0.0769
1.10	−2.8217	−2.4746	−2.1084	−1.7935	−1.5351	−1.3227	−1.1058	−0.9288	−0.7810	−0.6555	−0.4522	−0.2937	−0.0591
1.20	−2.8308	−2.4499	−2.0735	−1.7579	−1.5013	−1.2911	−1.0769	−0.9021	−0.7562	−0.6321	−0.4314	−0.2746	−0.0426
1.30	−2.8284	−2.1422	−2.0387	−1.7237	−1.4693	−1.2615	−1.0498	−0.8771	−0.7329	−0.6103	−0.4119	−0.2569	−0.0273
						D_2; HT ($\lambda^* = 1.223$)							
0.70	−2.8607	−2.6126	−2.2694	−1.9481	−1.6772	−1.4529	−1.2234	−1.0364	−0.8805	−0.7482	−0.5346	−0.3685	−0.1232
0.80	−2.9318	−2.6079	−2.2328	−1.9033	−1.6324	−1.4102	−1.1839	−0.9996	−0.8462	−0.7160	−0.5058	−0.3422	−0.1006
0.90	−2.9679	−2.5867	−2.1923	−1.8600	−1.5907	−1.3711	−1.1480	−0.9665	−0.8153	−0.6871	−0.4800	−0.3186	−0.0802
1.00	−2.9799	−2.5564	−2.1509	−1.8185	−1.5519	−1.3350	−1.1151	−0.9163	−0.7872	−0.6607	−0.4564	−0.2971	−0.0617
1.10	−2.9757	−2.5215	−2.1102	−1.7793	−1.5156	−1.3016	−1.0846	−0.9082	−0.7612	−0.6364	−0.4347	−0.2773	−0.0447
1.20	−2.9606	−2.4845	−2.0707	−1.7423	−1.4817	−1.2705	−1.0564	−0.8822	−0.7371	−0.6139	−0.4146	−0.2590	−0.0289
1.30	−2.9381	−2.4466	−2.0325	−1.7072	−1.4497	−1.2412	−1.0299	−0.8579	−0.7145	−0.5928	−0.3957	−0.2419	−0.0141
						DT ($\lambda^* = 1.095$)							
0.70	−3.0478	−2.6866	−2.2851	−1.9416	−1.6628	−1.4358	−1.2056	−1.0188	−0.8634	−0.7318	−0.5195	−0.3543	−0.1108
0.80	−3.0839	−2.6585	−2.2382	−1.8931	−1.6170	−1.3932	−1.1667	−0.9829	−0.8301	−0.7006	−0.4916	−0.3289	−0.0889
0.90	−3.0910	−2.6210	−2.1915	−1.8476	−1.5749	−1.3543	−1.1314	−0.9504	−0.8000	−0.6724	−0.4665	−0.3061	−0.0692
1.00	−3.0798	−2.5795	−2.1462	−1.8052	−1.5360	−1.3188	−1.0991	−0.9209	−0.7726	−0.6468	−0.4436	−0.2853	−0.0513
1.10	−3.0571	−2.5367	−2.1029	−1.7655	−1.5000	−1.2858	−1.0694	−0.8936	−0.7473	−0.6232	−0.4225	−0.2661	−0.0348
1.20	−3.0273	−2.4939	−2.0617	−1.7282	−1.4664	−1.2552	−1.0417	−0.8682	−0.7238	−0.6012	−0.4030	−0.2483	−0.0195
1.30	−2.9930	−2.4518	−2.0226	−1.6931	−1.4348	−1.2265	−1.0157	−0.8445	−0.7018	−0.5807	−0.3846	−0.2316	−0.0056
						T_2 ($\lambda^* = 1.00$)							
0.70	−3.1706	−2.7237	−2.2867	−1.9317	−1.6495	−1.4215	−1.1914	−1.0051	−0.8503	−0.7192	−0.5080	−0.3437	−0.1014
0.80	−3.1799	−2.6816	−2.2349	−1.8819	−1.6036	−1.3793	−1.1532	−0.9699	−0.8178	−0.6888	−0.4809	−0.3190	−0.0802
0.90	−3.1663	−2.6350	−2.1853	−1.8358	−1.5616	−1.3410	−1.1185	−0.9382	−0.7884	−0.6614	−0.4564	−0.2968	−0.0610
1.00	−3.1392	−2.5873	−2.1383	−1.7934	−1.5231	−1.3060	−1.0868	−0.9092	−0.7616	−0.6363	−0.4341	−0.2765	−0.0436
1.10	−3.1042	−2.5403	−2.0940	−1.7535	−1.4875	−1.2736	−1.0576	−0.8825	−0.7368	−0.6135	−0.4135	−0.2577	−0.0274
1.20	−3.0648	−2.4944	−2.0522	−1.7163	−1.4542	−1.2433	−1.0304	−0.8576	−0.7138	−0.5917	−0.3943	−0.2403	−0.0124
1.30	−3.0231	−2.4503	−2.0127	−1.6815	−1.4230	−1.2151	−1.0049	−0.8343	−0.6922	−0.5715	−0.3763	−0.2240	+0.0017

TABLE 2: REDUCED INTERNAL ENERGY

T^*/v^*	1.00	1.20	1.40	1.60	1.80	2.00	2.25	2.50	2.75	3.00	3.50	4.00	5.00
						H_2 ($\lambda^* = 1.729$)							
0.40	−0.7227	−2.5173	−2.7783	−2.6016	−2.3105	−2.0106	−1.6744	−1.3947	−1.1678	−0.9848	−0.7161	−0.5346	−0.3163
0.50	−0.8171	−2.6559	−2.8912	−2.6789	−2.3540	−2.0286	−1.6726	−1.3826	−1.1507	−0.9654	−0.6955	−0.5145	−0.2978
0.60	−1.0152	−2.7778	−2.9746	−2.7226	−2.3682	−2.0246	−1.6470	−1.3621	−1.1284	−0.9427	−0.6734	−0.4936	−0.2791
0.70	−1.1506	−2.8782	−3.0294	−2.7406	−2.3636	−2.0082	−1.6343	−1.3374	−1.1034	−0.9181	−0.6504	−0.4720	−0.2601
0.80	−1.2734	−2.9555	−3.0603	−2.7404	−2.3470	−1.9845	−1.6075	−1.3102	−1.0769	−0.8925	−0.6267	−0.4502	−0.2410
0.90	−1.3806	−3.0108	−3.0721	−2.7274	−2.3230	−1.9562	−1.5781	−1.2814	−1.0492	−0.8661	−0.6026	−0.4279	−0.2216
1.00	−1.4708	−3.0466	−3.0693	−2.7055	−2.2936	−1.9247	−1.5469	−1.2515	−1.0207	−0.8391	−0.5780	−0.4054	−0.2020
1.10	−1.5439	−3.0657	−3.0552	−2.6771	−2.2606	−1.8911	−1.5144	−1.2206	−0.9916	−0.8115	−0.5531	−0.3825	−0.1821
1.20	−1.6005	−3.0707	−3.0324	−2.6438	−2.2248	−1.8557	−1.4807	−1.1890	−0.9619	−0.7834	−0.5278	−0.3593	−0.1620
1.30	−1.6417	−3.0640	−3.0029	−2.6069	−2.1869	−1.8190	−1.4462	−1.1567	−0.9316	−0.7549	−0.5021	−0.3358	−0.1417
						HD ($\lambda^* = 1.412$)							
0.70	−2.6360	−3.7557	−3.5470	−3.0596	−2.5763	−2.1626	−1.7481	−1.4278	−1.1790	−0.9835	−0.7027	−0.5163	−0.2949
0.80	−2.7223	−3.7825	−3.5367	−3.0339	−2.5461	−2.1318	−1.7184	−1.3998	−1.1526	−0.9586	−0.6801	−0.4957	−0.2770
0.90	−2.7866	−3.7899	−3.5149	−3.0023	−2.5126	−2.0990	−1.6875	−1.3709	−1.1255	−0.9331	−0.6572	−0.4747	−0.2589
1.00	−2.8308	−3.7822	−3.4851	−2.9666	−2.4768	−2.0648	−1.6557	−1.3413	−1.0979	−0.9071	−0.6339	−0.4535	−0.2406
1.10	−2.8567	−3.7629	−3.4494	−2.9279	−2.4393	−2.0295	−1.6232	−1.3111	−1.0697	−0.8807	−0.6103	−0.4319	−0.2219
1.20	−2.8671	−3.7344	−3.4093	−2.8869	−2.4004	−1.9932	−1.5899	−1.2804	−1.0411	−0.8539	−0.5863	−0.4100	−0.2030
1.30	−2.8640	−3.6987	−3.3658	−2.8442	−2.3604	−1.9561	−1.5560	−1.2491	−1.0120	−0.8266	−0.5619	−0.3877	−0.1838
						D_2; HT ($\lambda^* = 1.223$)							
0.70	−3.4226	−4.1695	−3.7722	−3.1966	−2.6706	−2.2340	−1.8032	−1.4731	−1.2178	−1.0177	−0.7307	−0.5405	−0.3142
0.80	−3.4756	−4.1658	−3.7447	−3.1630	−2.6371	−2.2020	−1.7735	−1.4456	−1.1922	−0.9936	−0.7091	−0.3207	−0.2972
0.90	−3.5060	−4.1476	−3.7102	−3.1261	−2.6016	−2.1688	−1.7430	−1.4174	−1.1659	−0.9691	−0.6871	−0.5007	−0.2788
1.00	−3.5173	−4.1188	−3.6710	−3.0868	−2.5647	−2.1345	−1.7118	−1.3887	−1.1392	−0.9440	−0.6648	−0.4803	−0.2624
1.10	−3.5128	−4.0822	−3.6282	−3.0457	−2.5267	−2.0995	−1.6799	−1.3593	−1.1120	−0.9185	−0.6420	−0.4596	−0.2445
1.20	−3.4953	−4.0395	−3.5827	−3.0031	−2.4876	−2.0636	−1.6473	−1.3295	−1.0843	−0.8926	−0.6189	−0.4385	−0.2263
1.30	−3.4671	−3.9922	−3.5351	−2.9593	−2.4477	−2.0271	−1.6142	−1.2991	−0.0561	−0.8662	−0.5953	−0.4171	−0.2078
						DT ($\lambda^* = 1.095$)							
0.70	−3.8981	−4.3987	−3.8935	−3.2721	−2.7245	−2.2760	−1.8365	−1.5010	−1.2421	−1.0393	−0.7486	−0.5559	−0.3268
0.80	−3.9248	−4.3774	−3.8584	−3.2358	−2.6901	−2.2440	−1.8073	−1.4741	−1.2171	−1.0159	−0.7277	−0.5369	−0.3104
0.90	−3.9307	−4.3456	−3.8186	−3.1972	−2.6543	−2.2111	−1.7773	−1.4466	−1.1916	−0.9929	−0.7064	−0.5175	−0.2937
1.00	−3.9200	−4.3060	−3.7756	−3.1569	−2.6175	−2.1773	−1.7467	−1.4185	−1.1655	−0.9677	−0.6847	−0.4978	−0.2767
1.10	−3.8960	−4.2611	−3.7302	−3.1152	−2.5796	−2.1427	−1.7155	−1.3899	−1.1390	−0.9429	−0.6626	−0.4777	−0.2594
1.20	−3.8616	−4.2119	−3.6828	−3.0723	−2.5410	−2.1075	−1.6836	−1.3607	−1.1120	−0.9176	−0.6401	−0.4572	−0.2417
1.30	−3.8188	−4.1593	−3.6339	−3.0285	−2.5015	−2.0716	−1.6512	−1.3311	−1.0845	−0.8919	−0.6172	−0 4363	−0.2237
						T_2 ($\lambda^* = 1.00$)							
0.70	−4.2152	−4.5426	−3.9697	−3.3209	−2.7602	−2.3044	−1.8595	−1.5205	−1.2591	−1.0545	−0.7614	−0.5671	−0.3359
0.80	−4.2219	−4.5110	−3.9309	−3.2835	−2.7258	−2.2728	−1.8308	−1.4942	−1.2347	−1.0317	−0.7411	−0.5486	−0.3199
0.90	−4.2102	−4.4714	−3.8887	−3.2444	−2.6902	−2.2403	−1.8014	−1.4673	−1.2098	−1.0084	−0.7203	−0.5297	−0.3037
1.00	−4.1843	−4.4261	−3.8441	−3.2039	−2.6537	−2.2070	−1.7711	−1.4397	−1.1843	−0.9846	−0.6991	−0.5104	−0.2871
1.10	−4.1475	−4.3766	−3.7976	−3.1623	−2.6162	−2.1730	−1.7406	−1.4117	−1.1583	−0.9604	−0.6775	−0.4908	−0.2702
1.20	−4.1022	−4.3240	−3.7496	−3.1196	−2.5780	−2.1382	−1.7094	−1.3831	−1.1318	−0.9356	−0.6555	−0.4707	−0.2529
1.30	−4.0501	−4.2688	−3.7002	−3.0760	−2.5390	−2.1029	−1.6775	−1.3540	−1.1049	−0.9104	−0.6330	−0.4503	−0.2353

TABLE 3: REDUCED INTERNAL SPECIFIC HEAT

T^*/v^*	1.00	1.20	1.40	1.60	1.80	2.00	2.25	2.50	2.75	3.00	3.50	4.00	5.00
						H_2 ($\lambda^* = 1.729$)							
0.40	−1.4949	−1.4432	−1.2655	−0.9619	−0.6245	−0.3372	−0.0901	0.0520	0.1275	0.1657	0.1922	0.1941	0.1820
0.50	−1.4692	−1.3140	−0.9843	−0.5929	−0.2674	−0.0489	0.1027	0.1734	0.2037	0.2149	0.2151	0.2061	0.1859
0.60	−1.4048	−1.1169	−0.6851	−0.2944	−0.0322	0.1133	0.1985	0.2303	0.2390	0.2380	0.2265	0.2126	0.1884
0.70	−1.2966	−0.8881	−0.4201	−0.0780	0.1141	0.2065	0.2509	0.2614	0.2588	0.2514	0.2337	0.2170	0.1906
0.80	−1.1531	−0.6599	−0.2054	0.0734	0.2082	0.2634	0.2827	0.2807	0.2716	0.2606	0.2391	0.2207	0.1928
0.90	−0.9885	−0.4512	−0.0385	0.1794	0.2706	0.3007	0.3040	0.2941	0.2809	0.2675	0.2436	0.2241	0.1951
1.00	−0.8163	−0.2698	0.0896	0.2551	0.3139	0.3267	0.3193	0.3043	0.2883	0.2733	0.2476	0.2272	0.1974
1.10	−0.6470	−0.1162	0.1879	0.3105	0.3454	0.3460	0.3312	0.3126	0.2946	0.2784	0.2514	0.2303	0.1998
1.20	−0.4868	0.0121	0.2642	0.3522	0.3693	0.3610	0.3408	0.3196	0.3001	0.2828	0.2550	0.2334	0.2023
1.30	−0.3394	0.1186	0.3240	0.3845	0.3881	0.3732	0.3490	0.3257	0.3051	0.2873	0.2585	0.2364	0.2048
						HD ($\lambda^* = 1.412$)							
0.70	−0.9740	−0.3816	0.0303	0.2172	0.2816	0.2958	0.2894	0.2755	0.2607	0.2468	0.2232	0.2046	0.1775
0.80	−0.7515	−0.1631	0.1671	0.2902	0.3210	0.3190	0.3032	0.2848	0.2676	0.2523	0.2289	0.2078	0.1799
0.90	−0.5389	0.0079	0.2625	0.3390	0.3477	0.3354	0.3136	0.2923	0.2735	0.2573	0.2310	0.2110	0.1824
1.00	−0.3464	0.1403	0.3307	0.3735	0.3672	0.3480	0.3221	0.2988	0.2789	0.2619	0.2348	0.2142	0.1851
1.10	−0.1778	0.2430	0.3811	0.3992	0.3823	0.3583	0.3295	0.3047	0.2838	0.2662	0.2384	0.2175	0.1878
1.20	−0.0327	0.3235	0.4196	0.4193	0.3947	0.3671	0.3361	0.3101	0.2885	0.2705	0.2421	0.2207	0.1906
1.30	0.0912	0.3874	0.4498	0.4356	0.4052	0.3749	0.3421	0.3152	0.2931	0.2746	0.2456	0.2240	0.1934
						D_2; HT ($\lambda^* = 1.223$)							
0.70	−0.6536	−0.0524	0.2305	0.3137	0.3239	0.3125	0.2918	0.2716	0.2539	0.2386	0.2140	0.1953	0.1686
0.80	−0.4111	0.1172	0.3143	0.3543	0.3461	0.3265	0.3011	0.2786	0.2596	0.2435	0.2179	0.1986	0.1714
0.90	−0.2025	0.2402	0.3712	0.3822	0.3623	0.3375	0.3089	0.2849	0.2649	0.2481	0.2218	0.2021	0.1743
1.00	−0.0287	0.3308	0.4119	0.4029	0.3750	0.3466	0.3159	0.2906	0.2699	0.2527	0.2257	0.2055	0.1772
1.10	0.1144	0.3990	0.4424	0.4192	0.3856	0.3546	0.3222	0.2961	0.2747	0.2571	0.2296	0.2090	0.1803
1.20	0.2317	0.4515	0.4663	0.4326	0.3949	0.3619	0.3282	0.3012	0.2794	0.2614	0.2334	0.2125	0.1833
1.30	0.3282	0.4930	0.4856	0.4440	0.4031	0.3686	0.3338	0.3063	0.2840	0.2657	0.2372	0.2160	0.1863
						DT ($\lambda^* = 1.095$)							
0.70	−0.3876	0.1449	0.3221	0.3491	0.3355	0.3140	0.2881	0.2658	0.2472	0.2315	0.2069	0.1884	0.162[3]
0.80	−0.1557	0.2717	0.3773	0.3756	0.3511	0.3248	0.2959	0.2721	0.2526	0.2363	0.2109	0.1920	0.1654
0.90	0.0307	0.3605	0.4153	0.3952	0.3634	0.3338	0.3029	0.2780	0.2578	0.2411	0.2150	0.1957	0.1685
1.00	0.1785	0.4248	0.4432	0.4104	0.3736	0.3418	0.3094	0.2837	0.2629	0.2457	0.2191	0.1994	0.1718
1.10	0.2958	0.4730	0.4647	0.4229	0.3826	0.3491	0.3155	0.2891	0.2678	0.2503	0.2232	0.2031	0.1750
1.20	0.3895	0.5104	0.4822	0.4337	0.3908	0.3558	0.3213	0.2943	0.2726	0.2548	0.2272	0.2068	0.1782
1.30	0.4651	0.5401	0.4968	0.4433	0.3983	0.3622	0.3269	0.2994	0.2773	0.2592	0.2312	0.2104	0.1814
						T_2 ($\lambda^* = 1.00$)							
0.70	−0.1768	0.2656	0.3676	0.3632	0.3378	0.3116	0.2834	0.2603	0.2414	0.2257	0.2012	0.1830	0.1575
0.80	0.0330	0.3612	0.4070	0.3832	0.3504	0.3210	0.2906	0.2664	0.2468	0.2306	0.2055	0.1869	0.1608
0.90	0.1941	0.4275	0.4350	0.3984	0.3609	0.3292	0.2974	0.2723	0.2521	0.2355	0.2098	0.1908	0.1642
1.00	0.3178	0.4755	0.4562	0.4110	0.3701	0.3369	0.3037	0.2779	0.2572	0.2403	0.2141	0.1947	0.1676
1.10	0.4139	0.5118	0.4732	0.4218	0.3784	0.3437	0.3098	0.2834	0.2623	0.2450	0.2183	0.1986	0.1710
1.20	0.4896	0.5403	0.4875	0.4314	0.3861	0.3504	0.3157	0.2887	0.2672	0.2497	0.2225	0.2024	0.1744
1.30	0.5502	0.5635	0.4998	0.4402	0.3933	0.3567	0.3213	0.2939	0.2721	0.2542	0.2266	0.2062	0.1778

TABLE 4

CALCULATED AND EXPERIMENTAL CRITICAL CONSTANTS

	λ^*	T_c^*	v_c^*	p_c^*	
H_2	1.729	0.85	2.48	0.115	Calc.
		0.8917	4.33	0.0636	Exp.
HD	1.412	0.96	2.27	0.180	Calc.
		0.9705	4.15	0.0729	Exp.
D_2; HT	1.223	1.04	2.12	0.244	Calc.
		1.034	3.99	0.0811	Exp.
DT	1.095	1.08	2.07	0.265	Calc.
		(1.07)	(3.87)	(0.0862)	Exp.
T_2	1.00	1.10	2.03	0.282	Calc.
		(1.10)	(3.78)	(0.0901)	Exp.
Classical	0.00	1.30	1.77	0.434	Calc.
		1.28	3.09	0.121	Exp.

critical constants for the H_2 isotopes are of the same order of accuracy as are the calculated critical constants of the classical cell model. To illustrate this, the calculated and experimental critical constants of the five H_2 isotopes have been normalized by dividing by the calculated and experimental critical properties for a classical liquid and the resulting values are plotted in Figure 1. The agreement is surprisingly good. Departures from a linear dependence of the calculated critical constants on λ^* are to be noted for DT and T_2. Whether this does indeed occur or whether it indicates a deficiency in the model must wait until experimental critical constants are available for these two liquids.

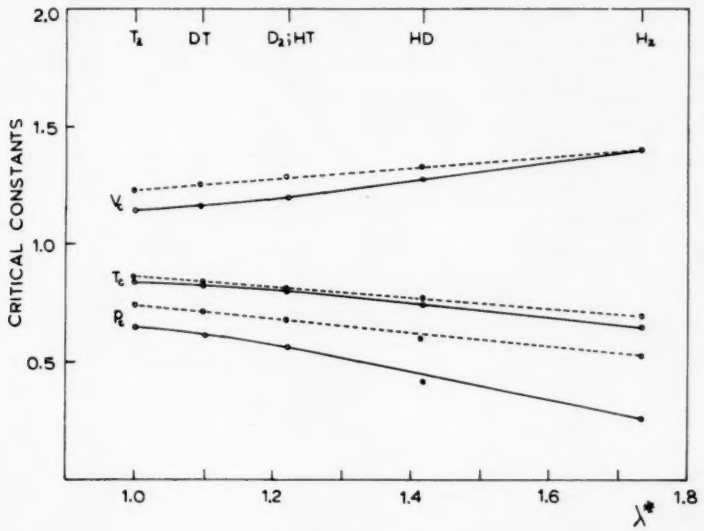

FIG. 1.—Normalized critical constants of the five H_2 isotopes. The solid lines join the calculated values of the critical constants and the dotted lines join the experimental values.

In addition to the usual assumptions of the cell model and the assumed validity of the simplified potential well, three other assumptions have been made. First, it has been assumed that all the H_2 isotopes have the same potential parameters. However, Michels *et al.*[7] have found that there is a difference between the potential

parameters of H_2 and D_2 which appears to be due to differences in polarizability between H_2 and D_2.[8] In addition, the fact that HD, HT, and DT rotate around a center of gravity which is not situated halfway between the two atoms, as is the case with H_2, D_2, and T_2, may cause further differences between the potential parameters. Secondly, the separation of translational and rotational motion is not completely justified, since the molar volume of liquid parahydrogen is known to be larger than that of liquid orthohydrogen.[9] Finally, it should be pointed out that Boltzmann statistics have been used throughout. In view of the relative crudity of the cell model it does not seem useful to correct for the differences in potential parameters or for the effect of rotational motion. However, it might be of interest to investigate the effect of applying quantum statistics. Strictly speaking, quantum statistics should not be used in the cell model. However, the introduction of quantum statistics very probably involves the same type of assumptions as does the introduction of the communal entropy into the classical cell model. Indeed, Prigogine and Philippot[10] have considered the effect of quantum statistics in liquid helium by allowing for the effect of doubly occupied cells.

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*EVIDENCES OF THERMAL SWEATING IN THE FOOT PAD OF THE CAT**

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The sweat pads of the cat have been considered to be in sweating behavior analogous to the palms and soles of man. Responding readily to sensory and mental stimuli, the latter are sluggishly if at all responsive in thermoregulatory sweating. Kuno[1] would deny them any thermoregulatory function, but states that sweating in the cat foot pad can be provoked by thermal agency, which led him to conclude that the sweating of the cat does not exactly correspond to the sweating of man. In the cat, Hasama[2] has caused sweating by application of heat to the

sweat centers. Magoun, Harrison, Brobeck, and Ranson[3] mention the appearance of sweat on the foot pads of some cats at the peak of the panting response to local heating of the brain. Sperling and Koppanyi[4] induced sweating by placing the animal in a hot box or by heating the carotid arteries.

The suggestion, therefore, is that the sweat glands of the cat foot pad do partake in the thermoregulatory response. Experiments to be discussed herein confirm the suggestion. The sweating response to increased body temperature is centrally controlled, there being evidence that nonnervous thermal sweating does not occur.[5] How effective foot pad sweating could be in temperature regulation is problematical, considering the small surface area involved. Nevertheless, the mechanism is present. Presumably, panting would be the major means for heat loss.

Activity of sweat glands has been studied by observation of potential changes and impedance changes across the foot pad.[6] To record these changes zinc-zinc sulfate electrodes have been placed one upon the surface of the central foot pad, the other subcutaneously at a short distance. These led to a direct-coupled amplifier and oscillograph for recording of potential changes, or to an impedance bridge with

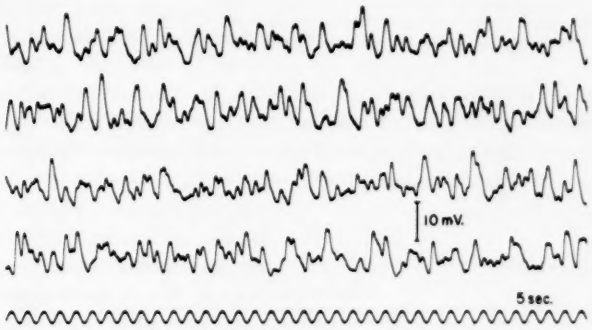

FIG. 1.—Spontaneous electrical potential activity of innervated sweat glands of the cat's foot pad.

condensor-coupled amplifier and oscillograph employed as detector. The animals were anesthetized with a mixture of chloralose (40 mg/kg) and urethane (0.8 gm/kg) administered by intraperitoneal injection. Body temperature was manipulated with the aid of a heating pad and ice bags, and was measured rectally with a thermistor thermometer.

Temperature and Spontaneous Potentials.—The sweat glands deprived of central control are electrically silent in the absence of stimulation; not so, the innervated glands. Spontaneous potential variations of the sort to be seen in Figure 1 were discovered by Hermann and Luchsinger[7] and have been studied recently by Ladpli and Wang.[8] In amplitude the spontaneous activity varies from preparation to preparation dependent apparently upon the level of anesthesia. That seen in Figure 1 is of exceptionally high amplitude.

Figure 2 illustrates the manner in which the spontaneous potentials of the foot pad are influenced by temperature change. It is noteworthy, as seen in the top three tracings, that no change in the level of spontaneous activity occurs in the range

between 37.5° and 39.5°C. There is then a relatively sudden increment in the level of spontaneous activity as seen in the fourth tracing taken at 40.5°C.

That there is no change with temperature in the range from 37.5° to 39.5°C indicates that the level of spontaneous activity is not simply related to body temperature. There is rather a critical temperature at which the idling central control mechanism is thrown (in a manner of speaking) into high gear.

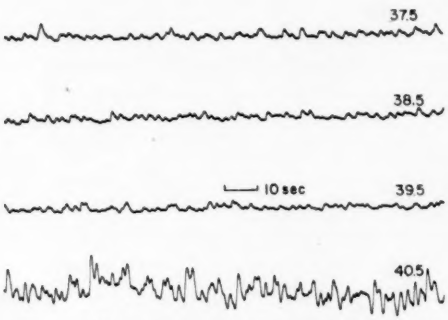

FIG. 2.—Spontaneous electrical potential activity of the innervated sweat glands of the cat's foot pad at four different rectal temperatures given for each record at the right in °C.

Temperature and Impedance.—Figure 3 presents the result of an experiment in which impedance across the foot pad was measured as body temperature was progressively increased. There was a slight decrease in impedance as temperature was increased from 37° to 40°C. This is the usual but not invariable result. As temperature exceeded 40°C there was a sudden drop in impedance to a very low value—so low, in fact, that one can conclude from other evidence[9] that the sweat was literally pouring from the foot pad.

The denervated foot pad exhibits with rise in temperature the slight decrease in impedance just as does the innervated foot pad. This change is a linear function of temperature. There is, however, no break in the impedance curve to indicate the occurrence of sweating even though the rectal temperature be increased to 43°C, at which temperature death may ensue. This provides another proof for the fact that nonnervous thermal sweating does not occur.[5]

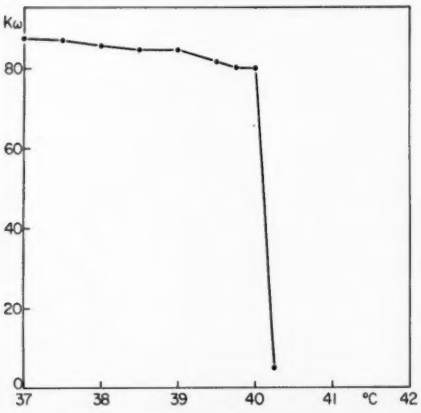

FIG. 3.—Impedance across the innervated cat's foot pad (ordinates) as a function of rectal temperature (abscissae).

Temperature and Galvanic Skin Reflexes.—If galvanic skin reflexes are elicited during change of temperature, an interesting effect is noted. Figure 4 illustrates this and provides further information on potential changes during warming and cooling. In the experiment illustrated, galvanic skin reflexes were elicited every 30 sec by stimulation of the central segment of the severed peroneal nerve. Record *A* shows that no change is encountered until the rising temperature approaches 39.5°C. There is then the increase in spontaneous activity already noted. In addition, the foot pad surface begins to become relatively positive and, as seen in *B*, the galvanic skin reflex, initially negative, reverses in sign to become a positive deflection.

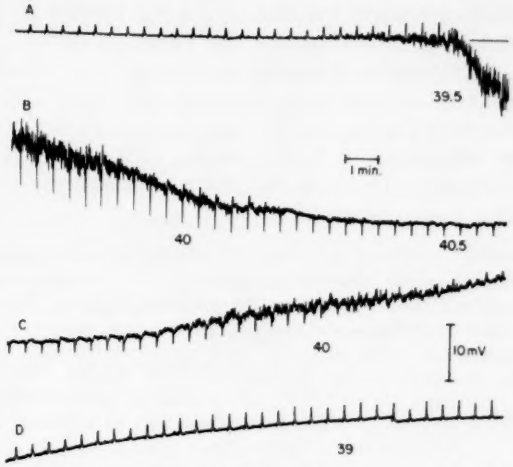

FIG. 4.—Samples from records to show the effect of temperature variation (rectal) on spontaneous activity and on galvanic skin reflexes elicited rhythmically (2 per min). Negativity of the foot pad surface upward. The initially negative rhythmic deflections are the evoked galvanic skin reflexes. Figures under each recording represent the rectal temperature.

Increase in spontaneous activity appears to take place within a fairly narrow range of temperature, for there is a secondary decrease as temperature approaches 40.5°C (record *B*) and a resurgence as temperature is lowered from that level (record *C*). On further cooling the normal type of record is restored (record *D*).

Figure 5 presents another similar experiment designed in the main to demonstrate that the reversal of galvanic skin reflex from negative sign to positive sign is centrally determined. Record *A* shows the normal reflexes elicited at 30 sec intervals. In record *B* the spontaneous activity is evident and the slowly developing relative positivity of the foot pad is taking place. Then in record *C* the positively directed galvanic skin reflexes are seen. Record *D* was made immediately after record *C*. It shows, at reduced amplification, a series of galvanic skin responses to motor nerve stimulation. Despite reversal of the reflex responses, the responses to motor nerve stimulation retain their normal negative direction. There is then no happening in the peripheral neuroglandular segment of the reflex mechanism to account for reflex reversal. It must, in the circumstance, be centrally determined.

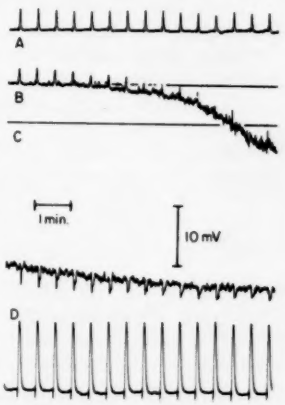

FIG. 5.—Effect of temperature variation on galvanic skin reflexes as in Fig. 4 except for record *D* which contains recordings of the galvanic skin response to motor nerve stimulation.

Two points of interest emerge from the findings presented in Figures 4 and 5. The first of these pertains to the reversal of the galvanic skin reflex. Being centrally determined, and since the arrival of impulses at the sweat glands causes a negative response, it seems as though the recording of positive responses must indicate

a deficit in centrally discharged impulses. This implies that an inhibitory reflex either is unmasked or is substituted for the excitatory reflex. Other evidence for the existence of inhibitory reflexes has been found in the manifestation of terminal rebound in reflexes engendered by stimulation of afferent C fibers.[10, 11]

The second point of interest is that the spontaneous activity which is increased by heating suffers a depression on further heating (Fig. 4 *B*). It is possible that this phenomenon is comparable to the depression of sweating of the palm and sole of man that occurs at high temperatures.[1, 12]

* This investigation was supported by a Public Health Service research grant number B-2816 from the National Institute of Nervous Diseases and Blindness, U.S. Public Health Service.

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MOLECULAR GENETICS OF AVIAN PROTEINS, II. CONTROL GENES AND STRUCTURAL GENES FOR EMBRYONIC AND ADULT HEMOGLOBINS*

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There is some contradiction in the literature concerning the properties and time of appearance of embryonic hemoglobin in the chicken. Hall[1] noted a progressive shift in the oxygen equilibrium curve of the hemoglobin as development proceeded; the principal change was a gradual decrease in oxygen affinity but no significant change in the magnitude of the heme-heme interactions. Saha[2] observed an al-

most hyperbolic oxygen equilibrium curve for 15-day-old embryo hemoglobin but a sigmoid oxygen equilibrium curve at hatching (20–21 days) and at 15 days after hatching; there was no significant change in the oxygen affinity. Saha[3] has also noted that 7-day chicken embryo "fetal hemoglobin" has a slightly more alkaline-resistant component than that present in the adult hemoglobin. Knight[4] identified immunologically two kinds of hemoglobin in the chicken, one being present already at two days of development and persisting in the adult, the other being identifiable only after the fifteenth day of development and also persisting throughout the subsequent life of the chicken. D'Amelio and Salvo,[5] expanding their earlier work, have shown both by starch and agar electrophoresis, as well as by immunological techniques, that there are two distinct embryonic hemoglobin components which comprise the only hemoglobin present at 68 hr of development: by 88 hr they found three additional hemoglobin fractions which are identical to those present in hemoglobin from adult chickens; by 11 days they reported that embryos had only traces of the embryonic hemoglobins and that the adult-type hemoglobins were the dominant respiratory blood proteins. Fraser,[6] using both electrophoresis and carboxymethyl-cellulose chromatography, resolved all chicken hemoglobin samples into two components; the only developmental difference reported was that the relative amounts of the two components varied; 5-day and 7-day embryos had 84 per cent of the more acid hemoglobin component and 16 per cent of the more basic hemoglobin component; the proportions changed gradually over the entire intra-egg period so that both newly hatched chicks and adult hens had 28 per cent of the acidic component—which Fraser[6] termed "embryonic hemoglobin"—and 72 per cent of the basic component—designated "adult hemoglobin." Wilt,[7] using agar gel, starch gel, and immunoelectrophoresis, observed two kinds of hemoglobin in the adult chicken—and these were also present in 2- and 3-day-old embryos; a third immunologically distinct globin-like component persisted only for 48 hr of development; this transient component behaved electrophoretically in the same way as the major adult hemoglobin component, although it was immunologically different.

A possible explanation for some of these different findings might be the "breed" or race of chicken used. This raises the interesting possibility that the presence or absence of embryonic hemoglobin and the time that its synthesis is "turned on" and "turned off" might be under separate genetic control—as suggested in regulation of adaptive enzymes in microorganisms,[8] certain alleles in maize,[9] and developmental protein sequences.[10] Thus, the authors have investigated the properties of the embryonic and adult hemoglobin of four different breeds of chickens, one strain of turkey, and the red-winged blackbird.

Materials and Methods.—Fertile chicken eggs were obtained from White Leghorns, New Hampshires, and Columbians kept on the University of Illinois poultry farm. Some eggs were incubated on the farm in forced air incubators; others were incubated in an oven in the laboratory. Hatching time was the usual 21 days, and a high proportion (90–100%) of eggs set under these conditions contained viable embryos at the time of sacrifice or healthy chicks at the time of hatching. Some small variation in the size of embryos during the first few days of development was noted, as also observed by others;[11] these variations are not significant with respect to the work presented here. Thirty fertile eggs from a flock of mixed "bantam" chickens were obtained from Dr. H. Shoemaker, Department of Zoology, University of Illinois; these chickens are derived from a panmictic population of various fancy bantam breeds and lay eggs in nests scattered about the vivarium grounds.

Fertile eggs of the California broad-breasted strain of turkey were purchased from Bonnett's Turkey Farm, Havana, Illinois. These were incubated in the same oven in the laboratory (and often at the same time) as the bantam eggs and some of the other chicken eggs. Red-winged blackbird (*Agelaius phoeniceus*) eggs were removed from nests of local birds and used the same day as collected. All embryonic "times of development" are expressed in days from the time the fertile eggs are placed in the incubator. As fertile poultry eggs are usually stored at a cool temperature for a few days prior to being incubated, there is apt to be a few hours' lag in actual embryonic age. Because of the use of wild populations of the red-winged blackbird, precise aging was not possible; by sampling different nests at one time, however, a graded series of embryo sizes was obtained, ranging from those showing first signs of hemoglobin synthesis (approximately 2 days old) to those ready to hatch (2-3 weeks).

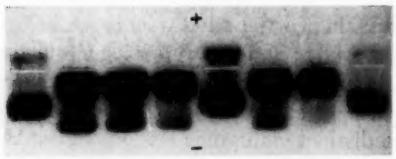

FIG. 1.—Starch gel electrophoresis of adult and early embryonic chicken hemoglobins. Phosphate gel, pH 7.2, 300 volts for 8 hr. From left to right: (1) adult chicken, (2) 4-day New Hampshire embryo, (3) 4-day Columbian embryo, (4) 4-day White Leghorn embryo, (5) adult chicken, (6) 4-day White Leghorn embryo, (7) 3-day White Leghorn embryo, (8) adult chicken. Note difference between embryonic and adult chicken hemoglobins, and absence of differences between breeds.

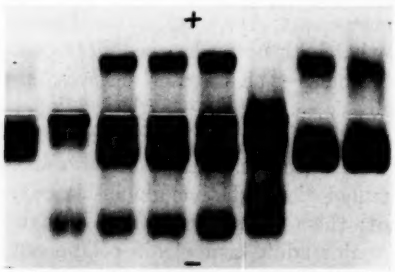

FIG. 2.—Difference in the time of activation of adult hemoglobin genes in the chicken and the turkey. Starch gel electrophoresis in borate buffer system, pH 8.2, at 250 volts for 12 hr. From left to right: (1) adult turkey, (2) 7-day turkey embryo, (3) 7-day White Leghorn chicken embryo, (4) 7-day New Hampshire embryo, (5) 7-day Columbian embryo, (6) 5-day Columbian embryo, (7) adult chicken, (8) adult chicken. Note that, while the 5-day-old chicken embryo and the 7-day-old turkey embryo have no adult hemoglobin, the 7-day-old chicken embryos have approximately half of their hemoglobin of adult electrophoretic properties. Although the major embryonic and adult hemoglobin components tend to overlap in this particular photograph, they can be completely resolved in longer electrophoretic runs, though with loss of sharpness of the minor hemoglobin bands.

Early embryos were dissected free from the yolk under isotonic saline (0.9 % NaCl), washed free of yolk by dipping gently into clean saline solution, and then bled into a small quantity of citrated and heparinized saline by severing the major blood vessels. The small amount of yolk contamination was completely removed by a combination of repeated filtration through glass wool and differential centrifugation. Larger embryos were bled from the heart, after first removing the extraembryonic membranes and washing the embryo with saline. Adult birds were bled by heart puncture. Because of the small quantities of hemoglobin needed in much of the work, it was customary to use single specimens, except for the 3-, 4-, and 5-day stages when three to six embryos were pooled. Time of hemoglobin change was determined by sacrificing embryos each day for the 2–7 day stages and then sampling every two days until the time of hatching. Hemoglobin first appears in turkeys 12–24 hr later than it does in chickens; thus, the first sampling of turkey embryos was done at $3^1/_2$ days. Electrophoretic experiments with individual early embryos, as well as with adults, showed no individual hemoglobin variation. For the "fingerprinting" comparison six dozen 5-day-old embryos were pooled so that enough hemoglobin could be obtained for purification by ammonium sulfate fractionation (45–55% saturation, based on 70.6 gm of ammonium sulfate to 100 ml of hemoglobin solution at 0°C being 100% saturation).

Erythrocytes were washed three or four times in large volumes of cold isotonic sodium chloride. Any trace of a "buffy coat" (leucocyte layer) was discarded. Lysis was accomplished by addition of a fixed volume of distilled water (for most purposes 5–10 ml of water to one ml of centrifugally packed erythrocytes). Toluene (Matheson, Coleman, and Bell, special purity grade) was stirred over the crude hemolysate to effect complete separation of hemoglobin from other cellular components. At this time, unless the samples were to be used for oxygen equilibrium, the hemoglobin was converted to the carbon monoxide derivative for increased stability. Cellular

debris was removed by centrifugation for one hour at $35-40 \times 10^3 g$. All of these preparation steps were done at 0°–4°C. Starch gel electrophoresis showed that over 95% of the soluble protein in these unpurified hemolysates was hemoglobin; histochemical staining of starch gels indicated traces of esterases and lactic acid dehydrogenases, which are removed upon purification.

Vertical starch gel electrophoresis,[12] as modified slightly by the authors,[10, 13] was used in screening all hemoglobin samples. Particularly good results were obtained with borate gels (pH 8) and phosphate gels (pH 7) run in a cold room at 0°C and at 250–300 volts for 8–15 hr. "Fingerprinting" was done by the method of Katz, Dreyer, and Anfinsen.[14] Best results were obtained with tryptic digests of deionized, heat-denatured purified hemoglobin.[15] Oxygen equilibria were measured spectrophotometrically as described elsewhere.[16] To ensure that any differences in electrophoretic behavior or in oxygen equilibria were intrinsic to the hemoglobins themselves and not due to differences in ionic or molecular environment, hemoglobin samples were purified by ammonium sulfate fractionation and—as recommended by Allen, Wyman, and Smith[17] in the comparison of Hb A and Hb F—were dialyzed against a common buffer ($\mu = 0.3$ or 0.6, potassium phosphate of specified pH, in the range 6.5 to 7.4).

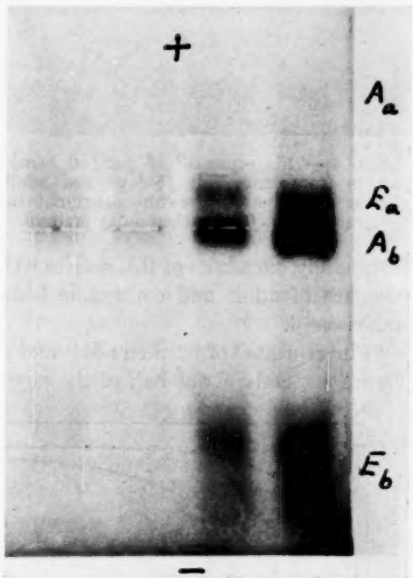

FIG. 3.—Simultaneous presence of adult and embryonic hemoglobins throughout development of the red-winged blackbird. Two samples on the left are from 2-day-old embryos shortly after the beginning of erythropoiesis; two samples on the right are from embryos just prior to hatching. Hemoglobin components have been homologized with those in developing chickens and turkeys; "A_a" is the more acidic adult hemoglobin component, "E_a" is the more acidic embryonic component; "A_b" is the more basic adult component; and, "E_b" is the more basic embryonic component.

Results.—Typical results of starch gel electrophoresis in phosphate and borate buffer systems are shown in Figures 1 and 2. Early embryos have a distinctly different hemoglobin from that of late embryos or adults. No difference is seen between the various breeds of chickens. Up to the fifth day of incubation only the two embryonic hemoglobin components are visible; but, by the seventh day approximately half of the hemoglobin is of the adult type. In further agreement with D'Amelio and Salvo[5] only traces of embryonic hemoglobins remain after 11 days. No difference in the time of "switchover" from embryonic to adult hemoglobin synthesis was seen between White Leghorns, New Hamsphire Reds, and Columbians; however, bantams appeared to lag by 12–24 hr in the time of appearance of the adult hemoglobin components, though their hemoglobins are electrophoretically and structurally (by "fingerprinting") identical with those of other chicken breeds.

Turkeys (see also Fig. 2) have adult and embryonic hemoglobins that are electrophoretically very similar to the corresponding hemoglobins of the chicken. However, the time of "switchover" is later and adult hemoglobin does not appear until $8^1/_2$ days of incubation; by 11 days half of the hemoglobin is of the adult type.

Red-winged blackbirds of all developmental stages show four hemoglobin bands, two of which seem to correspond in position to chicken and turkey embryonic hemoglobin and the other two of which correspond to adult hemoglobin (Fig. 3). Un-

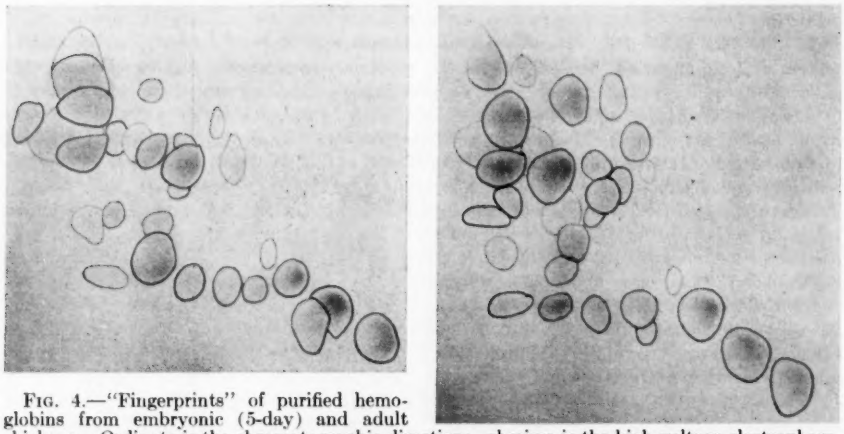

FIG. 4.—"Fingerprints" of purified hemoglobins from embryonic (5-day) and adult chickens. Ordinate is the chromatographic direction; abscissa is the high voltage electrophoretic direction. *Left:* Chicken 5-day embryo. *Right:* Chicken adult.

fortunately, no adults of this species were sampled; thus, whether the simultaneous presence of adult and embryonic hemoglobins continues past juvenile stages is unanswered.

"Fingerprints" of purified adult and embryonic chicken hemoglobin are shown in Figure 4. Only about half of the peptides appear to be in common and the general difference in the adult and embryonic chicken hemoglobin patterns slightly exceeds that for Hb A and Hb F,[18] known to differ from each other by 38–42 amino acids in one polypeptide chain (β versus. γ chain), though having the α chain in common.[19]

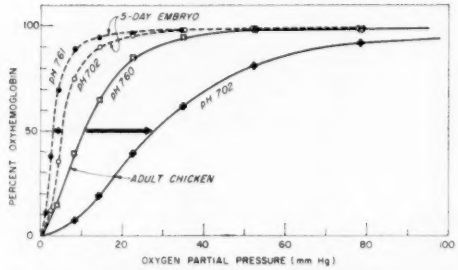

FIG. 5.—Oxygen equilibrium of purified embryonic (5-day) and adult chicken hemoglobins. Samples at comparable pH's have been dialyzed against a common potassium phosphate buffer (μ = 0.6). Arrows indicate the magnitude of the Bohr effect. Hemoglobin concentrations at 4%; temperature = 25°C.

Oxygen equilibria of purified and dialyzed hemoglobin from 5-day chicken embryo and adult chicken are shown in Figure 5. In agreement with Hall[1]—but not with Saha[2]—there is almost no difference in the magnitude of the heme-heme interactions, although the oxygen affinity of the embryonic hemoglobin is much greater than that of the adult hemoglobin. In addition, the Bohr effect is considerably less for embryonic chicken hemoglobin.

Discussion.—A distinct embryonic hemoglobin in poultry: On the basis of three independent and reliable methods of biochemical comparison, there is no question but that early embryonic chickens and turkeys have an embryonic hemoglobin that is distinct from that possessed by late embryos and adult birds. The electrophoretic results are very similar to those of D'Amelio and Salvo.[5]

Multiple hemoglobins of adult birds: The only apparent difference between our

studies and those of D'Amelio and Salvo[5] concerns whether there are three rather than two hemoglobin components in the adult chicken. By chromatography or paper electrophoresis, several workers[20] have found one major (80%) and one minor (20%) hemoglobin in the chicken and other birds. However, Rodnan and Ebaugh[21] reported that, in addition to the basic major hemoglobin component, some chickens had one and others had two minor acidic hemoglobin components. The authors have noted no consistent individual variation in this regard. The older hemoglobin samples, allowed to stand for several days in the cold room or frozen and thawed too much, often show a broadening of the acidic minor component hemoglobin zone, frequently showing subdivision into two or more diffuse bands. In addition, under conditions of higher voltage (500 volts) in starch gels it is possible to fractionate the basic major component into two zones, obtaining a pattern very similar to that of D'Amelio and Salvo.[5] Three components are clearly obtained in continuous-flow curtain electrophoresis using a low ionic strength ($\mu = 0.02$), pH 7.5 potassium phosphate buffer. These have been isolated and "fingerprinted." The anodal and cathodal components have 17 peptides apparently in common, but the anodal has 7 and the cathodal 9 unique peptides. The middle and predominant hemoglobin fraction has all 33 peptides (17 + 7 + 9). The middle component is an aggregation of the "anodal" and "cathodal" components. Thus, there must be at least two different kinds of polypeptide chains present. The number of peptides is suggestive that there might be more than two different polypeptide chains in adult chicken hemoglobin; possibly, as suggested by Saha and Ghosh,[22] "fast" and "slow" components have one polypeptide chain type in common and differ in the other. The variation between two or three components could be an expression of aggregation such as has been shown for "fast" and "slow" turtle (*Pseudemys scripta*) hemoglobins by Ramirez and Dessauer.[23] A further similarity between the situation in the turtle and in the chicken has been shown by "fingerprinting" the "fast" and "slow" turtle (*Pseudemys scripta*) hemoglobins;[15] each shows 27–29 peptides; "fast" turtle hemoglobin appears to have 10 unique peptides and 17 in common with "slow" turtle hemoglobin, the latter having 12 unique peptides. Besides the numbers, the general distribution and the pattern of peptides are quite similar between turtle and chicken hemoglobins, recalling suggestions that birds are feathered reptiles.[24] Studies on separation of the peptide chains of the various multiple hemoglobins and on genetic comparisons of closely related birds and closely related turtles should decide the actual number of active structural genes (cistrons) functioning in the adult animal.

Separate genetic control of the structural genes: Most significant is the presence in all red-winged blackbird embryos of hemoglobin components that are electrophoretically homologous to a mixture of adult and embryonic chicken hemoglobins (Fig. 3). This indicates that both adult *and* embryonic structural genes are activated from the very beginning of development, a condition resembling in certain ways the "high Hb F" trait in humans where both fetal and adult hemoglobins are found in normal adults. Neel[26] and Motulsky[27] have postulated that the "high Hb F" allele represents a mutation of the normal controlling gene that governs whether adult or fetal human hemoglobin will be produced by alternately activating and suppressing the beta chain and gamma chain cistrons. In addition, another type of control gene, comparable to a "tap gene," has been postulated to control the

amount of a given polypeptide chain that is produced; mutations of these "tap genes" are responsible for "α chain" and "β chain" thalassemias. While screening natural bird populations for genetic polymorphisms of hemoglobins and serum proteins, one individual pheasant, *Phasianus colchicus*, was found that almost but not entirely lacked the basic hemoglobin component which is usually the major hemoglobin fraction of adult birds (see Fig. 6). This bird was unable to produce the normal amount of the polypeptide chain that is unique to the "slow" hemoglobin component. Whether this is a genetic condition or the result of some unusual environmental trauma is unknown, but the condition does resemble a severe thalassemia, though unaccompanied by any embryonic hemoglobin compensation as occasionally occurs in the human.[28]

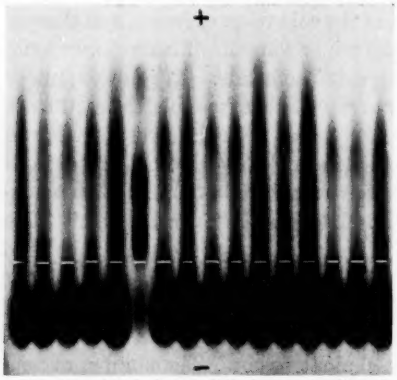

FIG. 6.—A "thalassemic" pheasant. Starch gel electrophoresis of hemolysates of 16 individual pheasants, *Phasianus colchicus*, collected in Livingstone County, Illinois. Note the sixth from the left, almost lacking the major hemoglobin component. Because of the age and concentration of the samples the minor hemoglobin component has spread out into an indefinite number of bands.

Recent studies by one of us[29] indicate that the tissue specific hemerythrins of sipunculid worms are the products of separate genes; this is based not only on "fingerprinting" studies, but also on analyses of genetic population polymorphisms. Thus, a separate control gene activates one hemerythrin cistron in erythrocytes located in the coelom and a second hemerythrin cistron in erythrocytes located in the vascular system. In addition, the existence of three different kinds of heterozygotes, varying in the amounts of "fast" and "slow" coelomic hemerythrins argues for the presence of separate "tap genes" regulating the amount of messenger released from the structural cistrons to which they are linked.

Therefore, it appears that separate control genes are a widespread phenomenon in animal—as well as in microbial[8] and in plant[9]—genetic systems. A hierarchy of control genes may be the fundamental explanation for differentiation in multicellular animals.[10]

Summary.—Early chicken embryos have a hemoglobin that is electrophoretically, structurally, and functionally different from that of late embryos and adults. Synthesis of embryonic hemoglobin is "turned off" after five days of incubation in White Leghorn, New Hampshire, and Columbian chickens; in bantam chickens the embryonic hemoglobin synthesis continues 12–24 hr later than in the other chicken breeds; in the turkey, adult hemoglobin begins to appear only after eight days of incubation. In the red-winged blackbird, adult and embryonic hemoglobins are synthesized together from the very beginning of erythropoiesis. Therefore, it is postulated that some kind of "control genes" exist which determine when—or whether—adult and embryonic hemoglobins are produced. In addition, there may also be control genes that regulate the total amount of a particular polypeptide chain that is produced; one individual pheasant was found with a condition resembling thalassemia in the human, which has been suggested to be due to a "tap gene" mutation.

We are especially grateful to Dr. Harold M. Scott and Mr. Walter McWard for supplying and incubating chicken eggs. Dr. Hurst Shoemaker kindly supplied us with "bantam" eggs and adults. We are indebted to Dr. Oliver Smithies and Otto Hiller, Department of Medical Genetics, University of Wisconsin Medical School, for helpful demonstrations and discussions of the starch gel electrophoretic technique. Dr. Charles G. Sibley, Department of Conservation, Cornell University, has been helpful in phylogenetic considerations of our work. Many individuals of the Illinois State Natural History Survey, including W. L. Anderson, J. A. Ellis, H. C. Hanson, J. A. Harper, and R. Labisky, have been most helpful in investigations on natural bird populations. We wish to thank Drs. S. Spiegelman and R. L. Watterson for a critical reading of the manuscript. These studies have been supported by grants from the U.S. Public Health Service (RG-7939) and the National Science Foundation (G-18082).

* Part I of this series appeared in *British Poultry Science*, **3**, 161, (1962).

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*REPRESSION OF ALKALINE PHOSPHATASE IN HUMAN CELL CULTURES BY CYSTINE AND CYSTEINE**

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Regulation of enzyme synthesis in microorganisms in many instances is controlled by the intracellular concentration of small molecular compounds.[1] Regulation may be positive in the sense that an inducer is required for synthesis, or negative in that synthesis occurs because of release from repression. For example, tyrosinase of Neurospora is regulated by both inductive and repressive mechanisms: an increase in the sulfur content of the medium represses tyrosinase synthesis and the enzyme is induced by aromatic amino acids.[2] A few examples of inductive and repressive effects have been described in cells of higher organisms in tissue culture.[3–7] Alkaline phosphatase in certain strains of mammalian cells is induced by adrenal glucocorticoid hormones or by a substrate of the enzyme, such as phenyl phosphate.[8–12] In general, skin fibroblasts are induced by substrates but not by hormone, whereas epithelial cell lines are induced by hormone but not by substrates. However, several lines of both epithelial and fibroblastic type are induced to form alkaline phosphatase by both hormone and substrate.[9, 12]

In microorganisms alkaline phosphatase synthesis is repressed by inorganic phosphate in the medium.[13] The enzyme in mammalian cells is independent of the concentration of inorganic phosphate.[10] However, alkaline phosphatase in certain inducible and constitutive mammalian cell strains is decreased on incubation in fresh growth medium, suggesting that a repressor may be present.[9, 10, 14] Other evidence for a repressor in fresh medium may be inferred from the following observations: (1) Medium in which cells have grown for at least 20 days is able to induce high alkaline phosphatase activity within a few days in skin fibroblasts.[9] The aged, inducing medium, is referred to as "conditioned" medium. (2) Substrate induction of most skin fibroblastic strains by phenyl phosphate requires 7 to 10 days.[9] However, when skin fibroblasts of an inducible strain are inoculated into medium containing phenyl phosphate in which other cells have been previously induced, a more prompt induction of alkaline phosphatase occurs within 3 to 5 days. (3) Dilution of "conditioned" medium with a small amount of fresh medium destroys its inducing activity.[9] These findings suggest that "conditioning" of medium both with and without phenyl phosphate involves removal of a repressor present in fresh medium. The following experiments indicate that L cyst(e)ine is the repressor or is converted to one.

Methods and Materials.—Cell lines: The methods used have been described previously.[9, 10] The skin cell strains are uncloned, diploid, human fibroblast-like cells established in culture following trypsinization of foreskin. The cultures were grown on glass surfaces in Waymouth's medium containing either 10% calf serum or 10% human serum.

Two HeLa lines were studied. "Ch" is a constitutive, prednisolone noninducible strain obtained from Mr. Lawrence Chessin and grown in Waymouth's medium with 10% calf serum. HeLa S_3, furnished by Dr. Harold Nitowsky, has low alkaline phosphatase activity which is inducible by prednisolone. It was grown in Waymouth's medium containing 10% human serum.

Cultural methods: (a) *Maintenance conditions:* The cells from several bottles were pooled and aliquots from this pool were inoculated into the number of bottles required for a particular experiment. To minimize variations in alkaline phosphatase activity between replicate bottles, the inoculum in most experiments was between 800,000 and 1,500,000 cells. Inocula of this size gave a confluent monolayer in 2 or 3 days. The medium was then decanted, and the monolayer was washed twice with Puck's saline.[15] "Maintenance medium" consisting of 10 ml of Puck's saline containing 10% calf serum was added to each bottle. In this maintenance medium there is no measurable net protein synthesis, although protein turnover is active as shown by the incorporation of radioactive leucine. To the bottles various vitamins, amino acids, and nutrient mixtures were then added to yield the final concentration of Eagle's minimum essential medium.[16] Cystine and cysteine were used also in the concentration found in Waymouth's medium. In all experiments equal numbers of replicate bottles incubated in maintenance medium without the addition of amino acids or vitamins served as controls. Alkaline and acid phosphatase and total cell protein of duplicate and triplicate bottles varied less than 20% when the cell monolayers had been grown for less than 5 days prior to the repression experiments described below. The alkaline and acid phosphatase activity of the cell strains varied somewhat between different experiments. Therefore, the results are expressed as a percentage of the specific alkaline and acid phosphatase activity of replicate control bottles incubated for the same interval in maintenance medium. (b) *Growth experiments:* In addition to maintenance conditions, experiments were carried out with growing 6- to 7-day-old replicate cultures of fibroblasts in Waymouth's medium. Three days before the cells were harvested the amino acid or amino acid mixture under study was added to the medium. Controls were unsupplemented replicate cultures grown for the same interval. Specific alkaline phosphatase activity of cultures grown in medium with added amino acids was compared to the specific alkaline phosphatase activity of the replicate controls. Pooled "conditioned" Waymouth's medium was prepared as previously described.[9]

Assay methods: The methods used in the preparation of deoxycholate lysates of cell cultures and the assay of alkaline and acid phosphatase activity have been described.[10] Protein determinations were carried out by the method of Lowry *et al.*[17] on an aliquot of the deoxycholate lysate.

Inhibition of alkaline phosphatase activity by L cysteine: Alkaline phosphatase from human kidney cortex was partially purified by a modification of Morton's method.[18] It showed 30-fold increase in specific activity. The partially purified kidney enzyme and crude alkaline phosphatase from deoxycholate lysed tissue culture cells were reacted at pH 8.1 and pH 8.8 with 2-fold dilutions of L cysteine in 0.1 *M* barbital buffer. The results were similar with deoxycholate lysates of cells and a partially purified kidney preparation. Following incubation of the enzyme and cysteine at room temperature for 5 min, substrate was added at various pH values and the final pH of the enzyme-cysteine-substrate mixture was measured. The activity of enzyme and cysteine mixtures was compared to the activity of the enzyme incubated in barbital buffer under the same conditions.

Results.—Repression of skin cell alkaline phosphatase under maintenance conditions: Table 1 shows that cultures incubated in maintenance medium for 40 to 48 hr double their alkaline phosphatase activity when compared to the enzyme activity of replicate cultures harvested at the beginning of the experiment. Replicate cultures which are continued in Waymouth's medium for 40 to 48 hr have at most 10 to 20 per cent increase in enzyme activity. The increase in alkaline phosphatase developed under maintenance conditions without measurable net protein synthesis, whereas the specific activity of acid phosphatase was unchanged. Additions of the components of Eagle's minimal essential medium to cultures in maintenance medium enhanced the alkaline phosphatase activity except in those cultures incubated with the 12 essential amino acids. Repression of alkaline phosphatase activity is observed in the presence of essential amino acids but not when a mixture of nonessential amino acids is added. Repression by essential amino acids is probably greater than our results indicate because protein turnover is stimulated by the

TABLE 1

REPRESSION OF ALKALINE PHOSPHATASE OF HUMAN SKIN FIBROBLASTS UNDER MAINTENANCE CONDITIONS BY L CYSTINE AND L CYSTEINE

Additions to maintenance medium	Number of experiments	Alkaline phosphatase*	Acid phosphatase*
Replicate cultures *before* adding maintenance medium	9	43	98
Replicate cultures incubated in maintenance medium alone for 40 to 48 hr		100	100
Eagle's vitamin mixture	3	140	114
Glutamine 2.0 mM	2	124	96
Mixture of 20 amino acids in concentration of Eagle's medium	1	30	118
Mixture of Eagle's 12 essential amino acids	19	25	119
Mixture of Eagle's 8 nonessential amino acids	5	170	106
Lysine, valine, threonine	3	195	124
Leucine, isoleucine, arginine	3	160	135
Phenylalanine, tyrosine, methionine	3	200	142
Cystine, histidine, tryptophan	4	35	134
Mixture of 9 essential amino acids (lacking cystine, histidine, tryptophan)	4	127	115
Mixture of 11 essential amino acids (lacking cystine)	6	140	121
L cystine 0.4 mM	4	15	88
L cystine 0.2 mM	1	20	75
L cystine 0.1 mM	2	75	97
L cysteine 0.8 mM	21	22	102
L cysteine 0.4 mM	3	25	86
L cysteine 0.2 mM	1	67	115

* Expressed as per cent of specific alkaline or acid phosphatase activity of replicate cultures incubated in maintenance medium alone.

amino mixture. When the 12 essential amino acids are added to maintenance medium in groups of three, only the mixture containing L cystine represses alkaline phosphatase. Cultures incubated in maintenance medium containing 11 essential amino acids but lacking cystine show no reduction in alkaline phosphatase activity. The repressing effect of the mixture of 12 essential amino acids, therefore, appears to be due to L cystine or L cysteine. L cystine in the concentration present in Eagle's medium (0.1 mM) has little repressing activity when used alone. However, as shown in Table 1, the presence of other essential amino acids greatly enhances repression of alkaline phosphatase when 0.1 mM L cystine is present. L cysteine 0.8 mM, the concentration in Waymouth's medium, and 0.4 mM cysteine repress alkaline phosphatase activity about 5-fold. L cystine and L cysteine in equivalent concentration with respect to the cysteine moiety are equally effective.

The acid phosphatase activity of cultures incubated in maintenance medium with addition of components of Eagle's minimal essential medium is slightly increased. This increase probably reflects the enhanced protein turnover. In contrast to alkaline phosphatase activity, acid phosphatase is not affected by adding L cystine or L cysteine to maintenance medium.

Repression of skin cell alkaline phosphatase in Waymouth's medium: Alkaline phosphatase is also repressed by L cyst(e)ine in growing cell cultures as shown in Table 2. Cystine 0.1 mM, the concentration in Eagle's medium, and cysteine 0.8 mM, the concentration present in Waymouth's medium, are effective repressors of alkaline phosphatase in growing cultures. Glutathione and Eagle's essential amino acid mixture also repress alkaline phosphatase presumably because of their cyst(e)ine content. Acid phosphatase activity was unchanged by growth in medium with L cyst(e)ine.

Although not as extensively studied, epithelial cell lines have a different response.

TABLE 2

REPRESSION OF ALKALINE PHOSPHATASE OF GROWING CULTURES OF HUMAN SKIN FIBROBLASTS IN WAYMOUTH'S MEDIUM BY L CYSTINE AND L CYSTEINE

Replicate cultures grown in Waymouth's medium. Three days before harvesting the cells the medium was supplemented with:	Number of experiments	Alkaline phosphatase*	Acid phosphatase*
No additions—control		100	100
Essential amino acids	2	20	100
Cysteine 0.8 mM	5	25	95
Cystine 0.4 mM	5	16	98
Cystine 0.1 mM	2	26	103
Glutathione 0.8 mM	3	35	99

* Expressed as per cent of specific alkaline or acid phosphatase activity of replicate cultures grown in Waymouth's medium with 10% calf serum.

TABLE 3

REVERSAL OF EFFECT OF "CONDITIONED" MEDIUM ON ALKALINE PHOSPHATASE PRODUCTION OF HUMAN SKIN FIBROBLASTS BY ADDITION OF L CYSTEINE

Replicate cultures grown for 48 hr in	Alkaline phosphatase*	Acid phosphatase*
Fresh Waymouth's medium	0.014	0.52
"Conditioned" medium	0.095	0.46
"Conditioned" medium with 0.8 mM cysteine added	0.013	0.48
"Conditioned" medium plus 11 essential amino acids (cyst(e)ine omitted)	0.095	0.44

* Specific activity expressed as mg of phenolphthalein released in 4 hr at 37°C by a deoxycholate lysate containing 0.1 mg of protein.

TABLE 4

CYSTEINE REPRESSION OF ALKALINE PHOSPHATASE OF HUMAN SKIN FIBROBLAST INCUBATED IN MAINTENANCE MEDIUM

Replicate cultures incubated in maintenance medium	Alkaline phosphatase specific activity*	Per cent control specific activity	Acid phosphatase specific activity*	Per cent control specific activity
Control—2 hr	0.197		0.55	
Cysteine 0.8 mM	0.154	80	0.53	96
Control—6 hr	0.175		0.61	
Cysteine 0.8 mM	0.200	114	0.60	98
Control—24 hr	0.410		0.62	
Cysteine 0.8 mM	0.220	54	0.65	105
Control—48 hr	0.510		0.58	
Cysteine 0.8 mM	0.110	22	0.53	91

* See footnote to Table 3.

An alkaline phosphatase constitutive, prednisolone noninducible HeLa strain "Ch," showed 40 to 60 per cent reduction in alkaline phosphatase activity when grown in Waymouth's medium supplemented with cysteine as described above. A "prednisolone" inducible HeLa strain S_3, on the other hand, has little alkaline phosphatase activity and this is not decreased by growth in Waymouth's medium with added cysteine. Prednisolone induction of alkaline phosphatase in this strain is as effective in the presence of added cysteine as in medium without added cysteine.

Effect of adding cysteine to "conditioned" Waymouth's medium: Table 3 shows that skin fibroblast cultures grown in "conditioned" Waymouth's medium for 2 days develop high alkaline phosphatase activity when compared to replicate controls grown in fresh Waymouth's medium. When L cysteine was added to "conditioned" medium, its capacity to permit synthesis of alkaline phosphatase was abolished. Addition of the 11 other essential amino acids had no effect upon "conditioned" medium.

Acid phosphatase activity was unaffected under any of the conditions listed in Table 3.

Kinetics of repression of skin cell alkaline phosphatase: Table 4 shows the kinetics

of repression by L cysteine of alkaline phosphatase of human fibroblasts incubated in maintenance medium. Little or no decrease in alkaline phosphatase activity is present at 2 hr or at 6 hr. At 24 hr alkaline phosphatase activity is reduced to approximately 50 to 70 per cent of the control value. The difference in alkaline phosphatase activity at 24 hr is due to a rise in alkaline phosphatase activity of the control cultures incubated in maintenance medium, whereas enzyme activity of the cultures incubated with added cysteine is unchanged. At 48 hr there is a marked decrease in alkaline phosphatase activity of the cultures incubated in the presence of cysteine. Control cultures incubated for 48 hr without cysteine show either a modest rise in alkaline phosphatase activity or no significant difference from the 24-hr enzyme activity. Acid phosphatase activity is unchanged.

Inhibition of alkaline phosphatase activity by cysteine: Sulfhydryl compounds are known to inhibit alkaline phosphatase activity of the crude and partially purified enzyme.[19, 20] However, pH values above 8.7 are required for marked inhibition unless high concentrations of cysteine (4.0 mM) are used. Table 5 demonstrates that the pH at which partially purified kidney enzyme and cysteine interact determines the degree of inhibition. As shown in Table 5, at pH 8.1 high concentrations

TABLE 5

INHIBITION OF PARTIALLY PURIFIED KIDNEY ALKALINE PHOSPHATASE BY CYSTEINE AND THE EFFECT OF pH ON INHIBITION

	Per cent of Enzyme Activity Remaining			
	Cysteine-enzyme interaction at pH 8.10		Cysteine-enzyme interaction at pH 8.85	
Cysteine Concentration	Final pH with substrate 8.7	Final pH with substrate 9.6	Final pH with substrate 8.5	Final pH with substrate 9.6
4.0 mM	54	55	15	14
2.0 mM	87	80	20	16
1.0 mM	90	100	45	44

of cysteine are necessary for inhibition while at pH 8.8 small amounts of cysteine are effective. Once the reaction between the enzyme and cysteine has occurred, it cannot be altered by subsequent changes in pH. These results suggest two different types of binding which are not interconvertible between cysteine and alkaline phosphatase dependent on the pH at the time the enzyme and cysteine interact. One interpretation is that at pH 8.1 the sulfhydryl group is undissociated and the binding between cysteine and the enzyme leads to only a slight reduction of alkaline phosphatase activity. At pH values above 8.8 the dissociated sulfhydryl combines with the enzyme to produce a marked inhibition of activity. The inhibition of alkaline phosphatase activity by cysteine both at pH 8.1 and pH 8.8 is partially reversible since 15 to 30 per cent of the inhibited enzyme activity can be restored by dialysis against Tris buffer at pH 7.8. In contrast to the partial reversibility of alkaline phosphatase activity inhibited by cysteine, the enzyme prepared from skin fibroblasts which had been repressed by cysteine showed no increase in activity following dialysis.

Discussion.—That cysteine-mediated repression of alkaline phosphatase is an interference with enzyme synthesis or activation and not sulfhydryl inhibition of the enzyme is suggested by the following evidence: (1) Kinetic studies show no difference at 6 hr in the alkaline phosphatase activity of cultures incubated in maintenance medium with or without L cysteine, even though cyst(e)ine enters the amino acid pool within 30 min and is concentrated to approximately twice the

medium concentration.[21-23] At 24 hr the enzyme activity of cultures incubated with cysteine is the same as in the 6-hr cultures. In contrast, replicate cultures in maintenance medium alone at 24 hr have a moderate increase in alkaline phosphatase. The enzyme is markedly repressed in cultures after 48 hr of incubation in medium with cysteine, whereas replicate cultures in maintenance medium alone show a modest continuing rise in enzyme activity. These kinetic data are more compatible with repression of alkaline phosphatase synthesis than inhibition of enzyme activity. (2) Inhibition of solutions or suspensions of alkaline phosphatase by cysteine requires either a high concentration of cysteine or pH above 8.8. Intracellular concentrations of cyst(e)ine that have been reported[21-23] would appear to be too low to inhibit enzyme activity significantly. On the other hand it is possible that slow inactivation of the enzyme at pH values below 8.8 in the presence of low concentrations of cysteine might be responsible for the long time period required before marked depression of enzyme activity occurs. (3) Inhibition of alkaline phosphatase activity by cysteine at pH 8.1 and at pH 8.8 is partially reversible by dialysis. However, dialysis does not increase the alkaline phosphatase activity of deoxycholate lysates prepared from cysteine-repressed fibroblast cultures. (4) Formation of HeLa S_3 alkaline phosphatase is not repressed in growing cultures by medium supplemented with cysteine even though the HeLa S_3 enzyme activity is markedly inhibited.

The mechanism responsible for cyst(e)ine-mediated repression of alkaline phosphatase is not known. It should be noted that in certain *E. coli* strains cyst(e)ine represses several enzymes necessary for uracil biosynthesis.[24] Low concentrations of uracil (1–2 μg) reverse the cysteine inhibition in bacteria.[24] Preliminary experiments indicate that the addition of 500 μg/ml uracil to our maintenance medium does not prevent alkaline phosphatase repression by cyst(e)ine.

A number of repressors of enzyme synthesis in bacteria also inhibit the activity of the enzyme. In *E. coli* the first enzyme unique to pyrimidine biosynthesis, aspartate transcarbamlyase, is repressed and inhibited by a cytosine derivative.[25] Similarly, arginine is both an inhibitor of the initial enzyme and a repressor of several enzymes involved in *E. coli* biosynthesis of arginine.[26] End-product inhibition of both enzyme activity and enzyme synthesis in bacteria appears to regulate and adapt cellular metabolism to the environment. It should be noted in this regard that whereas end-product inhibition is almost completely reversible upon dialysis, inhibition of mammalian alkaline phosphatase by cysteine shows partial reversibility under the conditions of our experiments.

In contrast to bacteria where a clear-cut relationship exists between enzyme and end-product repression, the relation of mammalian cell alkaline phosphatase to cyst(e)ine repression is obscure. Alkaline phosphatase content of cultured cells changes during growth and multiplication with the highest activity occurring during mitosis. The enzyme is concentrated on chromosomes, mitotic spindle fibers, and in the cytoplasm except for the equatorial plane.[10] Sulfhydryl compounds are important in mitosis, especially in the formation of spindle fibers and the mitotic apparatus.[27] It is possible that the effects of cyst(e)ine on alkaline phosphatase are important in controlling certain aspects of mitosis.

The restricted distribution of constitutive alkaline phosphatase among different adult tissues and the development of the enzyme under special conditions, such as

during wound healing and in some tumors, may be related to the release of a cyst(e)ine-mediated repression.

Summary.—Alkaline phosphatase of certain established human cell cultures is repressed by cyst(e)ine, a constituent of the usual growth media. Forty hours are required for substantial cyst(e)ine-mediated repression of alkaline phosphatase. The observations described suggest that repression is caused by interference with enzyme synthesis or activation rather than sulfhydryl inhibition of alkaline phosphatase activity.

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*CELL-FREE PROTEIN SYNTHESIS: THE ASSOCIATION OF VIRAL RNA AND E. COLI RIBOSOMES**

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The hypothesis that information specifying amino acid sequences in proteins is carried from the gene to ribosomes by messenger RNA[1] is now supported by an overwhelming body of experimental facts. Following the pioneer work on phage-infected bacteria,[2] the most convincing proof of the messenger hypothesis came from experiments utilizing synthetic polynucleotides to direct the synthesis of polypeptides in a cell-free system from *E. coli*.[3] Some time ago we observed that RNA from several plant viruses stimulated the incorporation of amino acids into protein in the *E. coli* system;[4] that specific protein can be made in this manner has been shown using RNA from the bacteriophage f2.[5]

Several plant viral RNA's have been characterized extensively, and our familiarity with one of them, TYMV-RNA, prompted us to use it to define the complex carrying out protein synthesis in the *E. coli* system. In this direction, three basic types of experiment have been carried out, all utilizing zone centrifugation through sucrose gradients.[6] In the first, viral RNA and *E. coli* ribosomes are mixed, fractionated on the gradient, and the RNA is located. Secondly, the RNA-ribosome mixture is fractionated, and the individual fractions are assayed for their ability to incorporate amino acids.[7] Finally, the RNA-ribosome mixture is allowed to incorporate amino acids, then fractionated, and the RNA and newly made protein are located. All three experiments give essentially the same result, namely, that in the cell-free system approximately 10 per cent of the ribosomes participate in protein synthesis, and the active complex consists of one 70s ribosome and one molecule of viral RNA. In this paper we present the results of experiments of the first kind.

Materials and Methods.—E. coli ribosomes were prepared as described previously.[4] TYMV was labeled with P^{32} by growing Chinese cabbage plants in Hoagland's solution No. 1 for several weeks,[8] then inoculating with virus, and transferring the plants to the same solution lacking phosphate, other than 0.1–1.0 mC $^{32}PO_4^{--}$ per liter. Six to ten days later the young leaves showing chlorosis typical of TYMV infection were harvested, minced, and the sap filtered through muslin. There followed a centrifugation at low speed to remove large debris, and a 90-min centrifugation at 40,000 rpm in the No. 40 rotor of the Model L Spinco to pellet the virus. The pellet was dissolved in a small volume of 0.01 *M* EDTA, and in each cup of the SW 39 rotor, 2.0 ml of this solution were layered on top of 3.0 ml of CsCl solution having a density of 1.30. After 3 hr at 35,000 rpm the virus (ρ = 1.49) was completely sedimented, while leaf protein, lipid, and polysaccharide contaminants floated, and could be decanted. The virus pellets were taken up in 0.05 *M* Tris buffer, pH 7.5, and freed of excess CsCl by another high-speed centrifugation. Finally, these pellets were taken up in 0.05 *M* Tris and shaken with water-saturated phenol as previously described.[9] This procedure routinely afforded RNA containing 10^6 cpm per mg.

Linear gradients of sucrose (5–20%) were formed, using a device like that described by Britten and Roberts.[6] After centrifugation the tubes were placed in a jig whose bottom consisted of a rubber stopper in which a syringe needle was embedded. The plastic tube could be forced down onto the needle, the stopper then forming a tight seal to the bottom of the tube. Drops emerging from the needle were collected in Shevsky-Stafford tubes, the volume and absorbancy of each fraction were measured, and then the entire fraction was transferred to ringed planchets, dried, and counted in a thin end-window gas flow counter.

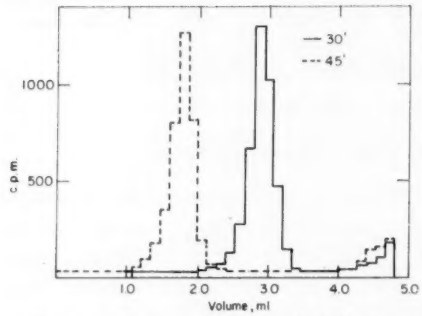

FIG. 1.—Resolution of the 5–20% linear sucrose gradient. Centrifugation of P^{32}-labeled TYMV (S = 115) at 35,000 rpm, 25°C for the times indicated.

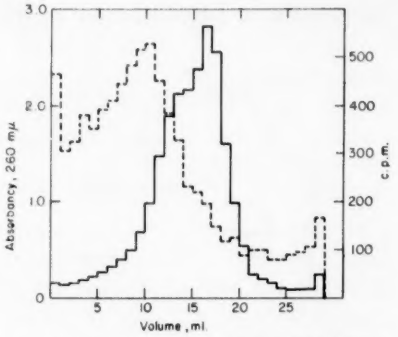

FIG. 2.—Attachment of TYMV-RNA to ribosomes. Centrifugation at 24,000 rpm for 2 hr at 25°C. 1.0 mg of ribosomes and 4 μg P^{32}-labeled RNA were applied to a gradient containing 0.01 *M* Tris, 0.01 *M* Mg^{++}. Solid line indicates absorbancy, dashed line radioactivity.

Figure 1 shows the result of centrifuging P^{32}-labeled TYMV for two different times. In each case the half-width of the zone of virus is approximately one tenth the total column height; presumably most of the broadening of the zone occurs during the layering of material onto the column and loading the rotor into the centrifuge. In the figures presented below, zones occupying more than one tenth the column height indicate heterogeneity with respect to sedimentation coefficient.

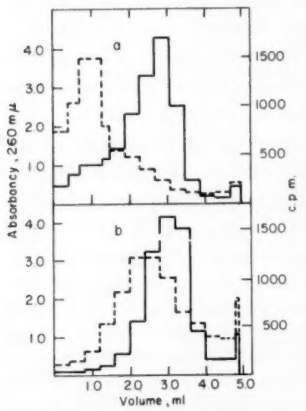

FIG. 3.—Effect of KCl on the RNA-ribosome complex. Centrifugation at 35,000 rpm for 45 min at 25°C. 400 μg ribosomes and 20 μg P^{32}-labeled RNA in 0.1 ml were applied to each gradient. Gradient in (*a*) contained 0.01 *M* Tris, 0.01 *M* Mg^{++}; in (*b*) contained 0.05 *M* Tris, 0.07 *M* KCl, 0.01 *M* Mg^{++}. Solid line indicates absorbancy, dashed line radio-activity.

Amino acid incorporation was measured in the complete system as described previously,[4] except that H^3-leucine and H^3-aspartic acid were used in place of the C^{14}-labeled mixture of amino acids. After incubation for 30 min at 36°C, the reaction was terminated by the addition of one-tenth volume of 50% TCA. The precipitates were collected by centrifugation and washed twice by solution in 0.1 *N* NaOH containing cold leucine and aspartic acid and reprecipitated with 5% TCA. The final pellet was dissolved in 1.0 ml of hyamine 10-x, taken up in 14 ml of toluene-PPO-POPOP scintillator fluid, and counted in a Packard liquid scintillation counter.

Results.—Viral RNA attaches to *E. coli* ribosomes in the ionic environment in which protein synthesis is observed; no factors other than Mg^{++} appear to be necessary for attachment. This is shown in Figure 2, in which a 0.2 ml zone containing ribosomes and P^{32}-labeled TYMV-RNA in 0.01 *M* Mg^{++}, 0.01 *M* Tris, pH 7.5 was analyzed on a gradient containing the same ionic composition. In this medium the ribosomes exist as a mixture of 70s and 100s particles; the P^{32}-labeled RNA is seen to move faster than 100s, and in fact occupies a region corresponding to 120–150s.

The distribution of ribosomes among sedimentation coefficient classes is affected by a number of factors in addition to Mg^{++} concentration. One of these is the

monovalent salt concentration; since we found initially that optimum amino acid incorporation was observed when 0.07 *M* KCl was present in addition to Mg^{++}, it was of interest to note the effect of KCl on viral RNA attachment.[4] In Figure 3*b*, the charge for the gradient was identical to that for 3*a*; the experiment differs only in the addition of 0.07 *M* KCl to the *gradient* itself. First, it can be seen that the sedimentation coefficient of the RNA-ribosome complex is decreased to 80–100s; secondly, the RNA:ribosome ratio of individual fractions is approximately twice that in the gradient lacking KCl. The calculation of the RNA:ribosome ratio for

TABLE 1

CALCULATION OF RNA:RIBOSOME RATIO

Fraction	μg RNA	A_{260} RNA	A_{260} Total	A_{260} Ribosomes	μg Ribosomes	$\frac{\mu g\ RNA}{\mu g\ Ribosomes}$	Molar Ratio
1	0.3	0.007	0.042	0.035	2.0	0.15	0.20
2	0.4	0.009	0.027	0.018	1.0	0.40	0.51
3	0.7	0.016	0.052	0.036	2.0	0.35	0.45
4	1.4	0.032	0.098	0.066	3.7	0.38	0.49
5	2.3	0.053	0.238	0.185	10.3	0.22	0.28
6	3.2	0.074	0.571	0.497	27.6	0.12	0.15
7	3.2	0.074	1.285	1.211	67.2	0.05	0.06
8	2.7	0.062	2.500	2.440	136.0	0.02	0.03
9	1.7	0.039	1.590	1.550	86.0	0.02	0.03
10	1.4	0.032	0.453	0.421	23.4	0.06	0.08
11	1.05	0.024	0.152	0.128	7.1	0.15	0.19
12	1.0	0.023	0.163	0.140	7.8	0.13	0.17

Data from Figure 3*b*. Each fraction was 0.4 ml. The extinction coefficient for TYMV-RNA was taken as 23, and for *E. coli* ribosomes as 18 for 1 mg/ml at 260 mμ. See text for details of calculation.

each fraction in Figure 3*b* is shown in Table 1. This was done by first determining the amount of RNA from the known specific activity of the RNA and the total counts for each fraction, converting to absorbancy units, subtracting the RNA absorbancy from the total, and dividing the remainder by the extinction coefficient for *E. coli* ribosomes. The weight ratio of RNA to ribosomes is given in the seventh column, and the molar ratio in the last. For conversion to the molar ratio, the molecular weight of TYMV-RNA was taken as 2.1×10^6, and of 70s ribosomes as 2.7×10^6. For fractions 5, 6, and 7 most of the absorbancy is contributed by ribosomes from the 70s region, rather than from the complex itself. Making a correction based on similar runs with ribosomes alone, the molar ratio for fractions 5, 6, and 7 is raised to 0.3, 1.0, and 0.4, respectively. Note that radioactivity attributable to complexes is distributed over half the gradient, a spread well outside the limits for a single species (0.1 of the column height, see Fig. 1). Comparison with Figure 3*a* suggests that the leading region (130-170s) is comprised of a small class of particles stable in the presence of KCl, while the peak region (80–100s) is comprised of those that have dissociated. Analytical ultracentrifugation of *E. coli* ribosomes in 0.01 *M* Tris, 0.01 *M* Mg^{++} shows a mixture of 100s and 70s particles; in the KCl-containing medium one finds 70s and 50s particles in the ratio of approximately 2:1.[11] Thus, the shift in the peak of radioactivity from 150s in Figure 3*a* to 80–100s in Figure 3*b* can probably be attributed to the dissociation of 100s particles. The RNA:ribosome ratio of 0.5 calculated for fractions 2, 3, and 4 in Table 1 is consistent with the interpretation that these fractions contain complexes composed of two 70s ribosomes and one molecule of RNA. Fractions 5, 6, and 7 (80–100s) should then contain complexes composed of one 70s ribosome and one molecule of RNA,

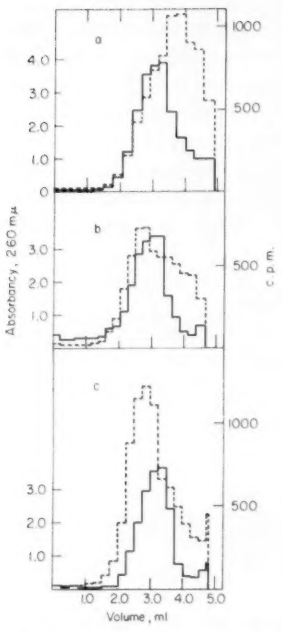

FIG. 4.—Stoichiometry of the interaction between TYMV RNA and *E. coli* ribosomes. Centrifugation at 35,000 rpm for 45 min at 25°C. Gradients contain 0.05 *M* Tris, 0.05 *M* KCl, 0.01 *M* Mg^{++}. To each was applied 0.1 ml containing 300 μg ribosomes and (*a*) 110 μg, (*b*) 42 μg, (*c*) 27 μg P^{32}-labeled RNA. Solid line indicates absorbancy, dashed line radioactivity.

and have an RNA: ribosome ratio of 1.0. Qualitatively this is observed, although the nature of the calculation precludes the assignment of an accuracy better than a factor of two to the ratio for these fractions. The conclusion that these complexes have the suggested composition rests primarily on their low sedimentation coefficient and the comparison between gradients with and without KCl.

Systematic variation of the RNA:ribosome ratio in separate gradients makes it possible to determine the stoichiometry of the interaction between ribosomes and RNA. In Figure 4*a*, *b*, and *c*, three experiments are shown in which the molar ratio of RNA to ribosomes is 0.48, 0.18, and 0.12, respectively. By comparing the distribution of P^{32} in such gradients with the distribution observed when viral RNA is run alone under identical conditions, an estimate of the fraction of RNA bound can be obtained. Figure 5 shows a plot of the fraction of RNA bound as a function of the RNA:ribosome ratio. It can be seen that saturation is obtained at a ratio of approximately 0.1. If one measures amino acid incorporation into protein as a function of the RNA:ribosome ratio in the complete system,[4] the incorporation goes through a maximum at approximately the same ratio, as shown also in Figure 5. This result indicates that only those ribosomes which function in protein synthesis bind viral RNA, and that by either test only 10 per cent of the ribosomes in the cell-free system are functional.

The attachment of viral RNA to ribosomes is reversible. This can be demonstrated in several ways, of which one is illustrated in Figure 6. In this case ribosomes and RNA in 0.01 Mg^{++} were layered on a gradient containing 10^{-4} *M* Mg^{++}. In the latter medium 70s ribosomes are dissociated into 50s and 30s particles. When this occurs, the RNA falls off and is found at a position corresponding to 30s, the sedimentation coefficient of free viral RNA. Reversibility can also be shown by the ability of P^{32}-labeled viral RNA to replace

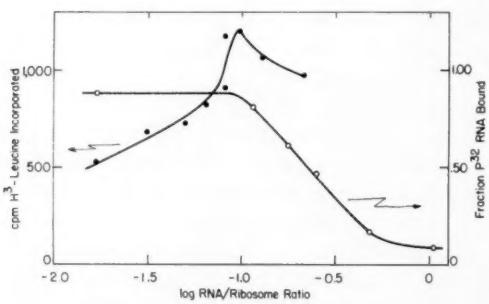

FIG. 5.—Correlation between RNA binding and amino acid incorporation. Open circles were obtained from six separate gradients as in Figure 4. The same cold RNA was used for isotope dilution in the gradients as was used in the amino acid incorporation assays, filled circles.

cold viral RNA bound to ribosomes. The reversibility is sufficiently complete that the order of addition of labeled and unlabeled RNA to ribosomes does not affect the fraction of label bound; the only pertinent variable is the final RNA:ribosome ratio.

It was of interest to determine which of the subunits of the 70s particle contains the binding site(s) for RNA. Accordingly, 50s and 30s particles were separated by centrifugation in a low Mg^{++} gradient, concentrated, and each mixed with viral RNA in high Mg^{++}. Gradient analyses of these interactions are shown in Figure 7*a* and *b*. This experiment has been performed four times with different preparations of ribosomes; in each case, attachment to 30s particles was observed, while in three of the four experiments attachment to 50s particles was observed. The preparation of 50s particles which did not bind RNA was also unable to combine with 30s particles to form 70s particles, although they had been derived from 70s particles.[12] We have, on several other occasions, observed *irreversible* dissociation of 70s into 50s and 30s; as might be expected, such particles are inactive in the amino acid incorporation system as well as being unable to bind P^{32}-labeled viral RNA.

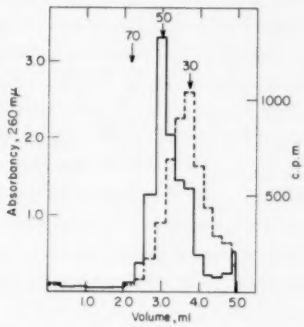

FIG. 6.—Dissociation of the RNA ribosome complex. Centrifugation at 35,000 rpm for 60 min at 25°C. 300 μg ribosomes and 7 μg P^{32}-labeled RNA in 0.1 ml of 0.01 *M* Tris, 0.01 *M* Mg^{++} was applied to a gradient containing 0.01 *M* Tris, 0.0001 *M* Mg^{++}. Solid line indicates absorbancy, dashed line radioactivity.

Discussion.—The existence of a cell-free system carrying out specific protein synthesis permits us to formulate two separate questions: what is the nature of the machinery in the cell-free system, and what relation does this machinery bear to that operative *in vivo*? It should be clear that the results cited above provide information concerning the first question only.

The first point to be made is that the active complex requires only Mg^{++} and such cofactors as are already present in washed *E. coli* 70s particles for its formation. This result has already been obtained for poly U^{13-15} and *E. coli* messenger RNA.[16] The contention that viral RNA requires energy to attach to ribosomes is not supported by our results.[13]

In 0.01 *M* Mg^{++} *E. coli* ribosomes exist as 70s and 100s particles. In this milieu the complex of viral RNA and ribosomes moves faster than 100s and might be comparable to the "heavy" 100s particles described by Risebrough *et al.*[17] The addition of KCl to the gradients (Fig. 3) demonstrates that, in the case of the viral RNA-ribosome complex, heavy 100s particles are artifacts in the sense that they are not obligatory for amino acid incorporation. Gilbert has recently shown that KCl dissociates 100s particles in the crude extract of *E. coli* as well, although in his

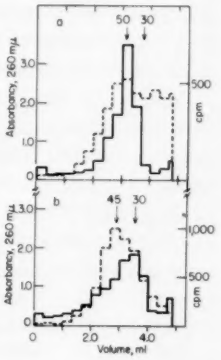

FIG. 7.—Attachment of TYMV-RNA to 30s and 50s particles. Centrifugation at 35,000 rpm, 25°C, for (*a*) 45 min and (*b*) 60 min. 0.1 ml containing (*a*) 250 μg 50s particles and 24 μg RNA and (*b*) 180 μg 30s particles and 48 μg RNA was applied to gradients containing 0.05 *M* Tris, 0.05 *M* KCl, and 0.01 *M* Mg^{++}.

experiment the *activity* for protein synthesis continued to move faster than 100s.[15] The sedimentation coefficient of the viral RNA-ribosome complex, together with the ratio of radioactivity to ultraviolet absorbancy in fractions from the complex region in KCl-containing gradients indicates that the complex contains at most two, and more likely one, 70s ribosome together with one molecule of viral RNA.

Published reports indicate that the active complex in reticulocytes *in vivo* consists of a string of ribosomes connected by a molecule of RNA.[18, 19] It is not yet clear whether a similar situation is found in bacteria, although Gilbert's experiment[15] suggests that. A polysome-like structure appears to be formed by poly U *in vitro*, but no such structure has yet been demonstrated with messenger or viral RNA. On the contrary, the present work suggests that viral RNA is incapable of forming such structures *in vitro*. Regardless of that handicap, polypeptide synthesis can be efficiently directed by viral RNA *in vitro:* we conclude that one 70s ribosome is sufficient machinery to carry out protein synthesis, a conclusion also reached by Gilbert.

Ishihama *et al.* reported that *E. coli* messenger RNA attached to 70s particles only when the particles were first dissociated in low Mg^{++} and then reassociated in the presence of the RNA.[16] We do not find this requirement for viral RNA. Ishihama *et al.* found that messenger RNA could attach to both 30s and 50s particles; we find the same true of viral RNA. Furthermore, an examination of the stoichiometry of association with the ribosomal subunits indicates that a given number of 30s or 50s particles is capable of binding nearly 80 per cent of the RNA bound by the same number of 70s particles. Since S-RNA attaches specifically to 50s particles,[20] and messenger attachment is only slightly enhanced by association with 30s particles, it is tempting to suggest another role for the 30s particle: could it be supplying the amino acid polymerase?

Summary.—Plant viral RNA associates with *E. coli* ribosomes in the absence of cofactors other than Mg^{++}. The complex thus formed appears to consist of one molecule of viral RNA and one 70s ribosome, and is capable of carrying out polypeptide synthesis. In a typical preparation of ribosomes, 10 per cent are capable of binding viral RNA; the same fraction functions in the amino acid incorporation assay. The association of viral RNA with ribosomes is reversed in 10^{-4} *M* Mg^{++}. Both 30s and 50s particles appear to contain site(s) for viral RNA attachment.

We should like to thank J. Ofengand for gifts of ribosomes and supernatant in the early stages of this work, L. Johnson for technical assistance, and E. P. Geiduschek for numerous helpful suggestions.

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THE MAINTENANCE OF THE ACCURACY OF PROTEIN SYNTHESIS AND ITS RELEVANCE TO AGEING

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The ways in which the accumulation of mutations might contribute to the process of ageing in higher organisms or in individual clones of cells has been discussed at length.[1] No corresponding treatment of the consequences of transcription errors in the translation of the DNA message into RNA and protein sequences seems to be available. Here I show that a consideration of the rate of accumulation of such errors leads to a paradox, the resolution of which may be relevant to the problem of ageing, and that there are a number of simple experiments which should decide whether this is the case.

The basic idea is a simple one, namely, that the ability of a cell to produce its complement of functional proteins depends not only on the correct genetic specification of the various polypeptide sequences, but also on the competence of the protein-synthetic apparatus. A cell inherits, in addition to its genetic DNA, the enzymes necessary for the transcription of that material into polypeptide sequences; the inheritance of inadequate protein-synthesizing enzymes can be as disastrous as the inheritance of a mutated gene. Similarly, a cell may deteriorate through a progressive decrease in the adequacy of its transcription mechanism, just as it may through the accumulation of somatic mutations.

As a basis for further discussion we shall suppose:[2] (*a*) that the sequence of amino acids in a protein is determined by the sequence of nucleotides in a corresponding region of the DNA; (*b*) that the immediate functions of DNA include the direction of the synthesis of transfer, microsomal, and messenger RNA but not of proteins; and (*c*) that the information for peptide sequence determination is carried by messenger RNA, but that other forms of RNA may affect the accuracy of protein synthesis.

There is little direct evidence concerning the accuracy of polypeptide-sequence determination. The accuracy of synthesis of messenger RNA is unlikely to exceed that of DNA replication, for which an error frequency of 10^{-8} per base has been estimated.[1] This would contribute an error frequency of about 3×10^{-8}

to protein synthesis if a degenerate three-letter code is assumed. However, the process of sequence determination also involves the specific reaction of the amino acids with their activating enzymes; this is likely to be a more important source of error. It must be difficult to distinguish a pair of amino acids as similar as valine and isoleucine even with an error level of 10^{-4}.

Our lack of knowledge of the error-frequency in protein synthesis makes it impossible to say, *a priori*, whether or not the accumulation of errors of protein sequence is relevant to the processes of ageing in higher organisms; this point must be decided experimentally. The nature of the mechanisms by means of which the accuracy of protein synthesis is maintained remains of interest, however low the error frequency. The first part of this paper will be concerned with these mechanisms.

We consider the following problem: suppose we could obtain a cell in which initially every polypeptide chain was in exact correspondence with its DNA sequence, the correspondence being assumed unambiguous. What would be the consequences of errors in protein transcription? We shall concentrate on what must be one of the main types of transcription error, namely, the replacement of a given amino acid by an incorrect amino acid. Let us roughly (and necessarily somewhat arbitrarily) divide the proteins of the cell into two sets, the first concerned with physical structure and intermediary metabolism, and the other concerned with the processing of genetic information, that is, the determination of the sequences of nucleic acids and polypeptides. The consequences of transcription errors for the two classes are very different.

If a small proportion of the protein molecules responsible for some metabolic function, say a part of glucose metabolism, were in error, then we might expect a somewhat lower *average* turnover number for the enzyme, a slightly reduced *average* specificity of the reaction, or perhaps a slight loosening of the *average* "control" of the reaction by feed-back inhibition, etc. These effects would not be cumulative; once the faulty messenger RNA or protein had been degraded, all memory of the error would soon be lost.

The situation is very different for the second class of proteins. Errors leading to complete loss of function would again affect only the efficiency of the information-transfer process in terms of average number of polymers replicated or synthesized per enzyme molecule. However, errors which lead to a reduced specificity of an information-handling enzyme lead to an increasing error frequency. Such processes are clearly cumulative and arguments which we shall next give in some detail suggest that, in the absence of an imposed selection for "accurate" protein-synthesizing units, must lead ultimately to an error catastrophe; that is, the error frequency must reach a value at which one of the processes necessary for the existence of viable cell becomes critically inefficient.

Under any given conditions the initial error frequency corresponding to the replacement of a particular amino acid in a particular position in a protein must be well-defined. Naturally it depends both on the amino acid replaced, the new and incorrect amino acid substituted (or, more correctly, on their base representations in the nucleic acid), and perhaps also on the environment in the peptide. Some replacements must certainly be more frequent than others on account of specificity at the transfer-enzyme level; further variety is introduced by any nonran-

dom distribution of errors of RNA synthesis. Similarly, each error in the protein-synthesizing system will induce a quite specific family of errors in the material synthesized; a loss of amino acid specificity of the phenylalanine activating enzyme, for example, could only effect substitutions involving the replacement of phenylalanine.

One feature of the dependence of the frequency of induced errors on the concentration of errors already present seems fairly certain, namely, that for small enough concentrations of errors the principal term is linear; that is, if we double the number of each kind of error already present in the protein-synthesizing system, we shall double the excess of induced errors over that in the initial error-free system. We shall not lose sight of the general features of the problem if, instead of considering the development of errors class by class, we lump them together and consider the error frequency p measured as the proportion of errors per amino acid present in polypeptide.

New protein synthesized by the initially correct enzymes will have some characteristic error concentration, say p_0. In the absence of any influence of errors already present on the frequency of error production, the error-level would settle down to p_0 once an amount of protein, large compared with that initially present, had been synthesized. If a slight linear dependence of the frequency of error production on the concentration of errors already present must be taken into account, we may, as a first approximation, write

$$\frac{dp}{dt} = \alpha p \tag{1}$$

where we consider the development of the system only after a time t_0 at which the error frequency p_0 has become established. This equation is only valid if p is small.

The solution of equation (1), $p = p_0 e^{\alpha t}$, shows that the error frequency initially increases exponentially and hence we predict the error catastrophe already mentioned. A proper treatment of this problem would take account of the time delay in the expression of errors. More importantly, the methods of probability theory should be used to determine the variance of p, etc.; the use of the differential equation (1) may be a rather poor approximation here, since the number of errors per cell may be small. These elaborations do not seem justified in the absence of more detailed experimental evidence.

How could an error catastrophe be avoided? Genetic selection for these proteins of DNA sequences, which in the course of transcription give particularly low error frequencies, may be possible within limits; selection for sequences which are completely inactivated by amino acid substitution would be more powerful. Selective scavenging of all incorrect proteins by hydrolytic enzymes might also contribute. However, in the light of recent work on ambiguous mutants,[3] it seems unlikely that these methods are powerful enough. What is needed is a selection based on the accuracy of protein synthesis, that is, a selection which rejects enzymes which lead to too many errors in protein synthesis. This could be achieved within a single cell only by a partial or complete segregation of the products of one piece of "protein-synthetic apparatus." All the available evidence argues against such segre-

gation. We must presume then that the selection works at a cellular or higher level.

In any population of cells there must be a certain variance in the accuracy of the protein-synthetic apparatus so that a sufficiently severe selective pressure could eliminate the least accurate cells; otherwise all cell lines would ultimately deteriorate. I shall try to put this result in a paradoxical form as follows: suppose we take a single bacterial cell and culture it under "ideal" conditions. After the first division we choose one daughter cell at random, allow it to divide again, choose one daughter cell at random, and proceed in this way in the absence of any "competition" between cells.[4] Then the arguments given above show that we finally get a nondividing cell even in the absence of mutation (naturally, the mutation frequency would also increase when the error frequency rose sufficiently). If these arguments are correct, one of the important functions of selection at cellular level is the maintenance of the accuracy of protein synthesis. Such a function would be additional to that usually discussed by geneticists and might quite possibly be equally important.

These speculations raise two important questions when applied to higher organisms. Firstly, does a cumulative inaccuracy in protein synthesis, connected with mutation only in so far as it is likely to lead to an increased somatic-mutation rate, play any role at all in the clearly extremely complex phenomenon of ageing? Secondly, what are the protective and selective mechanisms which prevent the error catastrophe in higher organisms?

There seems a fairly obvious experimental approach to the first of these questions. If we wish to study the effect of errors in protein synthesis in the absence of complications due to *primary* nucleic acid changes, we must increase such errors specifically, that is, without affecting nucleic acid synthesis. This could now be done by incorporating appropriate amino acid analogues. In the case of microorganisms of fairly representative selection of amino acid, errors could be induced by adding subtoxic concentrations of, say, *p* fluorophenylalanine, or, better, a mixture of *p* fluorophenylalanine and ethionine,[5] to the growth medium. A related experiment could almost certainly be done with mice. The level of incorporation of the analogue in these experiments should be far below that which causes death directly by gross inactivation of enzymes. In principle a pulse of analogue should produce consequences which cannot be reversed (except by selection) even after elimination of the analogue. It is perhaps worth remarking that it should be possible to study the effects of point mutations and primary errors of RNA synthesis by using bromouracil and fluorouracil, respectively, instead of amino acid analogues.

In higher organisms the situation is complicated by the existence of dividing and nondividing cells. The accumulation of transcription errors is likely to be particularly serious for the latter since, in the absence of division, selection at the cellular level is impossible. In slowly dividing tissue, selection may or may not be able to maintain the accuracy of protein synthesis; this can only be determined by experiment.

Now we come to the mechanism of protection or selection in higher organisms. If any part of the ageing process has to do with the accumulation of errors of polypeptide sequence, we need to know how it is that each new organism comes to have as clear a start as its parents. Separation of the germ line early may help in

some way, but we have no reason to believe that the *enzymes* initially present in the egg have been subjected to a significantly reduced possibility of error. Various solutions seem possible; for example, selection in the growing embryo may be strong enough or the process of embryogenesis may demand such a high accuracy that its successful completion guarantees the necessary accuracy of protein synthesis. One can also conceive of special mechanisms of quality control; for example, special proteins might be synthesized which are converted by a certain class of errors into lethal polypeptides. This would guarantee that the frequency of this class of errors in viable cells is kept low. At present there is no evidence available which enables one to select among these possibilities.

I wish to make it quite clear that I am not proposing here that the accumulation of protein transcription errors is "the mechanism of ageing." My object is the more modest one of pointing out one source of progressive deterioration of cells and cell lines. Since I am unable to estimate the time scale of this process, I can only suggest experiments which should show where, if anywhere, it contributes to the ageing process in higher organisms.

I am indebted to Professor H. C. Longuet-Higgins and Dr. F. H. C. Crick for valuable criticisms of my original manuscript.

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*DYNAMIC ROLE OF TRIPLET STATES IN PHOTOSYNTHESIS**

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The purpose of this communication is to point out some attractive features of primary photosynthetic steps based, partially at least, on *energy transfer* and *energy multiplication* mechanisms which involve the triplet state of chlorophyll. A more critical comparison of triplet state mechanisms, singlet state mechanisms, and photoionization mechanisms will be deferred until a later time. Here we strive only to correct some misunderstandings concerning the possible role of triplet states in photosynthesis and to speculate upon certain mechanisms often excluded from past thinking because of the misunderstandings.

Population of Triplets.—There seems little doubt that efficient singlet → triplet radiationless transitions (intersystem crossing) can occur in molecular aggregates, providing processes such as radiation or chemical quenching which limit the lifetime of the singlet state are not too fast. In the case where excitation transfer is

slow compared with the singlet-triplet radiationless transition time, the process is not fundamentally different from that which occurs for a molecule in an inert solvent[1-3] such as a hydrocarbon "glass." When the excitation transfer time is fast compared with the time for intersystem crossing, the probability of transfer divides itself among a number of molecules, but the over-all rate remains the same as for the localized case, providing the molecular vibrations are not affected by the fast excitation transfer. In the particular strong coupling limit[4] in the singlet where the vibrations of the aggregate in the excited state are different than they are for localized excitation, the singlet-triplet transition probability is different by virtue of its dependence on vibrational factors[2, 3] in the transition matrix elements. However, these factors and the associated transition probabilities are probably larger than they are when both excitations are localized, since the magnitude of vibrational factors between two nondegenerate electronic states often increases with increasing nonorthogonality of the vibrational eigenfunctions in the two states. Furthermore, the decreased energy separation between singlet and triplet states of the aggregate as compared to that in the isolated molecule should also enhance intersystem crossing.[5] Thus, we conclude that in almost all cases, except the unreasonable one where both singlet and triplet exciton interactions are in the strong coupling limit, the radiationless intersystem crossing process in a molecular aggregate should have the same order or higher transition probability than that normally found in rigid glass experiments. The arguments in the weak and intermediate coupling limits are particularly applicable to chlorophyll *in vivo* where absorption and fluorescence spectra imply that the intermolecular coupling is weaker than in crystalline chlorophyll.[6]

The expectation of efficient intersystem crossing in aggregates is borne out in mixed crystal experiments[7] with organic molecules. More important, a certain amount of information about intersystem crossing in aggregates of chlorophyll *in vivo* is implied from existing fluorescence quantum yield measurements. It is known that monomeric chlorophyll-*a* in ether or methanol solution under 430–436 mμ excitation shows a fluorescence quantum efficiency of about 33 per cent.[8] Similarly, recent measurements by Goedheer[9] using ~615 mμ excitation of *monomeric* chlorophyll-*a* both in $10^{-6}M$ methanol solution and in postetiolated bean leaves show nearly identical fluorescence quantum efficiencies, the *in vivo* to *in vitro* ratio running between 0.6–1.2 for a series of experiments. Such experiments indicate that the rate of intersystem crossing in monomeric chlorophyll-*a* *in vivo* or *in vitro* may be up to twice that for the purely radiative process from the singlet state. Even though the 67 per cent upper limit to the efficiency can be achieved only in the absence of other nonradiative processes, it appears that intersystem crossing might be an important path by which excited singlet states are depleted in monomeric chlorophyll-*a*.

In aggregated chlorophyll *in vivo*, efficient photosynthetic processes further reduce the lifetime of the excited singlet. Such an effect would be anticipated from the over-all high quantum efficiency of photosynthesis and is evidenced by the greatly reduced fluorescence quantum efficiency in mature plants. Taking 2 per cent to be the fluorescence quantum efficiency for chlorophyll aggregates *in vivo*[8, 9] and *assuming no change in the rate of intersystem crossing in the aggregate*, it would appear that at most only about 4 per cent of the singlets undergo intersystem cross-

ing to the triplet state. Such a low efficiency would hardly be important. If it actually existed in the photosynthetic system, the arguments presented in this paper would not be pertinent. On the other hand a 10-fold increase in the rate of intersystem crossing in the aggregate would enable triplet state population to be important even in the face of strong "chemical quenching" of the singlet state. As suggested in the first paragraph of this section, an increase of such magnitude may not be unreasonable.

Some evidence for an increased efficiency of intersystem crossing in *in vivo* chlorophyll aggregates does in fact seem to exist. Experiments[10] have shown that in living cells the normal 2 per cent fluorescence efficiency rises to 4 per cent upon inhibition of photosynthesis. Assuming that the radiationless transition from the excited singlet state directly back to the ground state is unimportant in the aggregate, this result implies that under inhibition of photosynthesis, where the excited states can do no photochemistry, 96 per cent of the excited molecules do indeed undergo intersystem crossing to the triplet state. The rate of intersystem crossing in the *in vivo* aggregate therefore appears to be about 24 times that for fluorescence radiation. In the case where photochemistry is not quenched by an inhibitor, use of the 2 per cent fluorescence efficiency leads one to expect quantum efficiencies of 48 per cent for intersystem crossing and 50 per cent for photochemistry from the excited singlet state.[11] These estimates are relatively crude because it has been assumed that inhibition changes only the efficiencies, but not the rates of the various processes which take place in the *in vivo* system. More important, this simple picture would be severely complicated by the presence of two quantum processes (*vide infra*) involving singlet or triplet states. Nevertheless, one is still tempted to say that a good fraction of the excited *in vivo* chlorophyll-*a* molecules at least reach the triplet state. In order for the triplets to contribute to photochemistry in the plant, they must do something useful during their $\lesssim 10^{-3}$ sec lifetime.[12, 13] Later in the paper we will point out that the triplets should indeed be able to do something very useful during this period of time.

One may at first think that the population of triplet states in a photosynthetic system should be accompanied by some phosphorescence (triplet-ground state emission) and might conclude that, since none has ever been observed, the existing evidence is therefore against triplet state population. There are three reasons why phosphorescence is not expected from *in vivo* aggregates of chlorophyll molecules, even though the triplets do get populated. The first reason has to do with the nature of monomeric chlorophyll in particular, the second concerns the behavior of triplet states in molecular aggregates in general, and the third has to do with the fact that, if efficient photochemistry from the triplet state is to occur, the triplets have little time to phosphoresce. The first two points will be discussed separately later in the paper under the headings of *Quenching of Triplets* and *Triplet-Triplet Annihilation and Energy Multiplication*. It develops that a low phosphorescence quantum yield need in no way be related to the efficiency of intersystem crossing to the triplet state.

Triplet Energy Transfer.—It has often been remarked that "the transfer of triplet energy in molecular aggregates is slow because the required overlap of electronic eigenfunctions in the intermolecular region must be small." This statement only partly hits the mark. The interaction energy β[14] for triplet excitation transfer in

hydrocarbons does depend primarily upon electron exchange[15] which, in turn, depends on intermolecular overlap of the wave functions. The interaction energy, however, need not be excessively small, being of the same order of magnitude as that for lowest singlet excitation transfer in certain π-electron molecules.[7, 16] The principal reason for this is that electronic eigenfunctions for a many-electron system fall off considerably less rapidly than $\exp(-Z_{eff}r/n)$ by virtue of the decrease toward unity of the effective nuclear charge Z_{eff} at large electronic distances r.[17] A direct experimental determination of the nearest neighbor β for crystalline benzene gave[16] 0.0015 e-v (12 cm^{-1}) corresponding to a time for one excitation transfer event of about 4×10^{-13} sec! Such a fast process can easily overwhelm other radiative and nonradiative processes from the triplet state.

Theoretically, the exchange interaction can be shown to be dependent upon the mutual orientation of the molecules,[16] the interaction of greatest magnitude occurring when the π-orbitals on each molecule are pointing directly toward one another. It is important to point out that the triplet interaction, unlike that for certain singlets,[18] has nothing whatsoever to do with the strength or polarization direction of the transition from the ground state to the triplet state. It would truly be coincidental if the optimum orientation for greatest heat of aggregation in the ground state, optimum orientation for singlet transfer, and that for triplet transfer were all the same. It should be realized, however, that even when the orientations are not optimum, as in crystalline benzene, energy transfer may still occur with high efficiency.

The relatively large triplet interaction energy is not the only reason that triplet energy transfer in molecular aggregates is important. The lifetime of the triplet state, being much longer than that of the singlet, allows ample time for triplet energy transfer even in the absence of large interaction energies. Thus, the two points together, moderate interaction energies and intrinsically long triplet state lifetimes, cause triplet energy transfer in many kinds of organic aggregates to be as important as singlet transfer, or more so.[19] There is therefore no obvious reason why the triplet state should not provide an efficient means of transferring energy through chlorophyll aggregates.

Another statement often made is that triplet energy transfer, being a short range interaction, dies off exponentially with distance, so that at most only nearest-neighbor transfer is ever expected. Again, this statement is only partially true. Certainly, for two interacting molecules in a vacuum, the exchange term for fixed Z_{eff} does drop off exponentially with distance at large distances. In real molecules the exchange term diminishes considerably more slowly than this, because Z_{eff} is itself decreasing. More important, in a molecular aggregate the transition probability for excitation transfer does not depend solely upon the direct (vacuum) interaction between two molecules. Any interactions which can occur by virtue of the fact that the molecules are both coupled to the whole system, and are thus indirectly coupled to one another, will contribute to the transition probability. In many cases the indirect interactions are expected to outweigh the direct ones by far.

Indirect interactions have been found to be responsible for long-range tunneling of triplet excitation between energy traps (sinks) in a host crystal when the triplet states of the host are nearby. The mean time for this transfer in the case where the aggregate is one dimensional is given by[20]

$$\tau \approx \hbar \Delta E^{N} \beta'^{-(N-1)} \beta^{-2}$$

for both β and $\beta' \ll \Delta E$, where ΔE is the energy difference between the triplet states of the guest and the host, β is the nearest-neighbor guest-host vibrational-electronic interaction energy, and β' is the nearest-neighbor host-host interaction. N is the number of intervening host molecules. The time τ is expected to be *concentration-dependent* because of the exponential factor N, and is expected to be *temperature-dependent* because of the fact that ΔE effectively decreases with increasing temperature. This mechanism provides a good way by which triplet states can undergo long-range energy transfer in molecular aggregates containing energy traps. In the instance where the energy traps are shallow or where there are no traps, the simple formula above does not apply. Nevertheless, in such cases an analogous type of long-range transfer of energy occurs. *Thus, it is possible for triplet excitation to migrate efficiently either through pure aggregates or through aggregates containing impurity or defect traps.*

Quenching of Triplets.—Radiationless processes (intersystem crossing) between a singlet state and a triplet state have been shown to depend upon the strength of the spin-orbit coupling between the two states and upon the magnitude of the vibrational Franck-Condon factors associated with the two states.[2, 3] The Franck-Condon factors range from zero to unity, and, for the latter value, singlet-triplet radiationless transitions between π-electron states of hydrocarbons take place in roughly 10^{-10} sec.[21] Such high rates would easily dominate fluorescence which occurs in only about 10^{-8} sec, and would even be able to compete successfully with the faster *in vivo* singlet state mechanisms in the photosynthetic system.

The Franck-Condon factors are very much smaller than unity for singlet and triplet states of widely different electronic energy, but approach unity as the two electronic states get close together. This point is especially pertinent in the process analogous to intersystem crossing which we call *triplet state quenching*, that is, the radiationless transition between the first excited triplet state and the ground state. One sees that *triplet states lying relatively close to the ground singlet are expected to be quenched rapidly by a radiationless transition to the ground state.* The over-all triplet state lifetime τ_T, which is the reciprocal of the sum of the triplet radiative and nonradiative rates, therefore decreases markedly for decreasing triplet state energy E_T. Furthermore, of the molecules reaching the triplet state, the fraction f_P[22] which phosphoresce is expected to decrease rapidly with decreasing E_T. The series anthracene[23] ($E_T = 1.82$ e-v, $\tau_T < 0.1$ sec, f_P = very low), naphthalene[24] ($E_T = 2.64$ e-v, $\tau_T = 2.6$ sec, $f_P \approx 0.16$), and benzene[24, 25] ($E_T = 3.66$ e-v, $\tau_T = 7.0$ sec, $f_P = 0.25$), illustrates this point for simple hydrocarbons. In chlorophyll-*b* Becker and Kasha[13] have found that the triplet state lies near 1.43 e-v (865 mμ). Such a low-lying triplet state is expected to be subject to rapid quenching. Similarly, the triplet state of chlorophyll-*a* lies very close to the ground state (1.40 e-v, 885 mμ).[26] The 10^{-3} sec triplet state lifetime[12, 13] of monomeric chlorophyll is therefore doubtless caused primarily by nonradiative transitions from the triplet state back to the ground state, and it is not unexpected that the quantum yield for phosphorescence emission is low. There is no reason to believe that, associated with chlorophyll in aggregates, there is not a similar quenching mechanism.

A low phosphorescence quantum efficiency for the monomeric chlorophylls has

indeed been indicated in the experiments of Becker and Kasha,[13, 26] a result which should not be construed to mean that the triplet state is not populated efficiently, but instead that the quenching rate as expected for the low-lying triplet state is high.

Triplet-Triplet Annihilation and Energy Multiplication.—In a molecular aggregate, phosphorescence and nonradiative quenching[27] directly back to the ground state are not the only ways by which triplet states can disappear. *Triplet excitations on two different molecules can mutually annihilate one another, so that one of the two molecules goes back to the ground state while the other is promoted to a higher electronic state having up to twice the energy of the triplet state!*[20] Spin selection rules allow this higher state to be a singlet, a triplet, or a quintet,[28] where, respectively, zero, two, or four electrons are unpaired.

The interaction energy for the triplet annihilation process, like the triplet transfer process, depends upon electron exchange.[20] Long-range triplet annihilation interactions through a vacuum are therefore not very strong. However, just as for long-range trap-to-trap triplet energy transfer in a molecular aggregate, the annihilation process itself can occur at long range, providing host states are relatively low-lying. The existence of excitation residing on nearest neighbors is therefore not an important requirement for efficient annihilation in such cases. Furthermore, it can also be shown[20] that, either in pure molecular aggregates or in aggregates containing a low concentration of shallow traps or a high concentration of deep traps,[30] the triplet energy transfer rate, as well as the annihilation rate, can both far exceed the rate at which radiation or simple quenching of the triplet state back to the ground state occurs. In other words, by virtue of the relatively long triplet state lifetime for radiation plus quenching, an efficient mutual annihilation of triplets in molecular aggregates can occur which allows triplets to be converted into high energy quanta. Thus, an important *energy multiplication* process can take place by way of the triplets. In pure organic crystals the annihilation process may serve only to quench phosphorescence rapidly;[20] in mixed organic crystals, long-range annihilation between traps can partially quench phosphorescence to produce higher singlets and lead to delayed fluorescence;[20] in a photosynthetic system, the mutual annihilation of triplets can lead to useful high energy photochemistry.

It must be borne in mind here that even though annihilation is a two-quantum process whose rate depends upon the square of the concentration of excited states, the dependency of the annihilation rate upon light intensity remains linear in the limit of fast annihilation. This fact follows from an examination of the over-all kinetics.[20] Physically it is an expected result since, in the limit where the major cause of triplet disappearance is bimolecular annihilation rather than unimolecular quenching, the stationary triplet concentration depends on the square root of the rate at which triplets are produced, i.e., on the square root of the light intensity; and the rate of triplet-triplet annihilation as well as the rates of processes which depend upon it, such as delayed fluorescence and high energy photochemistry, depend linearly on light intensity. The well-known linear variation of photosynthetic yield with light intensity in the range of low intensities up to saturation intensities[31] is therefore not at variance with the presence of a fast triplet-triplet annihilation step in the photosynthetic mechanism.

Summary.—In this communication the possible importance to photosynthesis of *triplet state quenching*, *triplet energy transfer*, *triplet-triplet annihilation*, and *triplet energy multiplication* has been pointed out. The more restricted role of triplets in photosynthesis discussed by Franck[32] and others[33] can now be broadened to include these more efficient processes. The presence of triplet mechanisms in the photosynthetic apparatus would not, of course, rule out quenching, energy transfer, annihilation, and energy multiplication involving singlet states; nor does it rule out annihilation and energy multiplication steps via either the triplet[20] or singlet states[34] to produce charge separation with further energy migration or chemical processes involving the charge carriers.[35] It would not be surprising if two or more of these types of energy transfer mechanisms combine in the primary photochemical steps. Such might be anticipated from Emerson enhancement effects[36–38] which indicate that chemical cooperation on a relatively long time scale (~5 sec) takes place between the products of primary light absorption at different wavelengths. Thus, two-quantum physical mechanisms may combine in a "two-quantum" chemical process in over-all photosynthesis.

The presence of shallow energy sinks in the photosynthetic apparatus seems assured by the existence of electronically similar but slightly different pigments.[36, 38] These energy sinks are chlorophyll-*a* molecules which appear to be in very special chemical environments or in very special states of aggregation. One might speculate that at room temperature efficient energy transfer and annihilation may occur *between the energy sinks themselves* (according to what was said earlier in the paper about long-range effects), provided the energy sinks are appropriately coupled through the nearby states of their own accessory pigment system.[39] However, at fairly low temperatures the transfer of energy may be retarded. At such temperatures where triplet energy transfer is relatively slow and where photochemistry has been quenched, triplet-triplet annihilation may reveal itself as delayed fluorescence having a nonexponential decay[20] of similar half life as the triplet state. That low temperature emission of this kind has already been in fact observed[41, 42] perhaps may be direct evidence for a dynamic rather than a static role of triplet states in photosynthesis.

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[3] *Ibid.*, in press.

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[5] McRae, E. G., and M. Kasha, *J. Chem. Phys.*, **28**, 721 (1958).

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[12] Livingston, R., and V. A. Ryan, *J. Am. Chem. Soc.*, **75**, 2176 (1953); Livingston, R., *ibid.*, **77**, 2179 (1955). The doing of something useful *in vivo* further shortens the lifetime of the triplet.

[13] Becker, R. S., and M. Kasha, *J. Am. Chem. Soc.*, **77**, 3669 (1955); see also ref. 26 of the present paper.

[14] The interaction energy referred to here is the transition matrix element for a radiationless transition between an initial state $\psi_A\psi_B\dagger$ and a final state $\psi_A\dagger\psi_B$, where the dagger represents electronic singlet or triplet excitation and the A and B represent two different molecules. The characteristic time for such a transition is given roughly by $\hbar\beta^{-1}$ where $\hbar$ is Planck's constant divided by 2π.

[15] Merrifield, R. E., *J. Chem. Phys.*, **23**, 402 (1955).

[16] Nieman, G. C., and G. W. Robinson, *J. Chem. Phys.*, in press.

[17] Slater, J. C., *Phys. Rev.*, **42**, 33 (1932).

[18] Foerster, Th., *Discussions Faraday Soc.*, **27**, 7 (1959).

[19] Assume as an upper limit that the singlet interaction in crystalline chlorophyll is given by the energy difference between the crystal absorption maximum at 720 $m\mu$ and the absorption maximum of the monomer at 660 $m\mu$.[6] This energy is at most only 100 times that for the triplet interaction and yet the lifetime of the singlet is certainly more than 100 times shorter than the triplet lifetime.

[20] Sternlicht, H., G. C. Nieman, and G. W. Robinson, *J. Chem. Phys.*, in press.

[21] Corresponding to a β of about 10^{-5} e-v (0.08 cm^{-1}); spin-orbit interactions between states surely fall within two orders of magnitude of this value.

[22] In the absence of quenching of the lowest singlet, $f_P = Q_P/(1\text{-}Q_F)$, where Q_P and Q_F are the quantum efficiencies for phosphorescence and fluorescence. In general $f_P = \tau_T/\tau_P$, where τ_P is the *radiative* triplet state lifetime, and τ_T is, as before, the over-all triplet state lifetime.

[23] Craig, D. P., and I. G. Ross, *J. Chem. Soc.*, **1954**, 1589.

[24] Gilmore, E. H., G. E. Gibson, and D. S. McClure, *J. Chem. Phys.*, **20**, 829 (1952); *ibid.*, **23**, 399 (1955).

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[26] Singh, I. S., and R. S. Becker, *J. Am. Chem. Soc.*, **82**, 2083 (1960).

[27] Quenching back to the ground state in a way described in the last section is, of course, but one of many ways that quenching can occur. Quenching by impurities and by chemical reactions are two other ways. Triplet-triplet annihilation described in this section is another more complicated way by which depletion of triplets can occur.

[28] The lowest quintet state of chlorophyll should not be overlooked. According to estimates of the same type made by Gouterman,[29] the lowest quintet state of chlorophyll should lie roughly in the range 2.34–3.27 e-v, an energy region about double that of the lowest triplet. Therefore (*vide infra*), the lowest quintet state appears to be accessible as a final state for triplet-triplet annihilation. Since the lowest quintet state is expected to have a lifetime (for intersystem crossing to lower triplets) possibly as long as 10^{-4} sec, relatively slow chemical steps or further annihilation steps can occur from the quintet state. Thus, while singlets or triplets having the same energy may not be able to carry out efficient photochemistry or annihilation because of their short lifetimes, the quintet by living longer might be able efficiently to carry out chemistry or annihilation at roughly double the energy of the lowest triplet. The author wishes to acknowledge a helpful discussion with George S. Hammond concerning quintet state chemistry.

[29] Gouterman, M., *J. Chem. Phys.*, **33**, 1523 (1960).

[30] *Shallow* and *deep* compare the electronic energy difference ΔE between guest and host with the interaction energy β for the radiationless transition. A deep trap is one where $\Delta E \gg \beta$, while a shallow one is where $\Delta E \lesssim \beta$.

[31] *See*, for example, French, C. S. in *Light and Life*, ed. W. D. McElroy and B. Glass (Baltimore: Johns Hopkins University Press, 1961), pp. 463–464.

[32] Franck, J., *Daedalus*, **86**, 17 (1955); *Research in Photosynthesis: Proceedings of the Second Gatlinburg Conference on Photosynthesis of the National Research Council, October 25–29, 1955*, ed. H. Gaffron *et al.* (New York: Interscience Publishers, 1957), pp. 19–30, 142–146.

[33] Rabinowitch, E., *J. Phys. Chem.*, **61**, 870 (1957); Kasha, M., *Rev, Mod. Phys.*, **31**, 162 (1959).

[34] Northrup, D. C., and O. Simpson, *Proc. Roy. Soc.* (*London*), **A244**, 377 (1958); Choi, S., and

S. A. Rice, *Phys. Rev. Letters*, **8**, 410 (1962) and *J. Chem. Phys.*, in press. However, annihilation of singlets can produce only ionization or short-lived singlet states but no triplets or quintets.

[35] See, for example, Calvin, M., *Light and Life*, ed. W. D. McElroy and B. Glass (Baltimore: Johns Hopkins University Press, 1961), pp. 317–355.

[36] French, C. S., *Carnegie Institution of Washington Year Book*, No. 58, 1959, pp. 318–323; see also ref. 31, pp. 458–463 and pp. 466–469; and also a forthcoming review by Smith, J. H. C., and C. S. French, *Ann. Rev. Plant Physiology*, 1963.

[37] Emerson, R., R. F. Chalmers, and C. Cederstrand, these PROCEEDINGS, **43**, 133 (1957).

[38] Rabinowitch, E., and Govindjee, in *Light and Life*, ed. W. D. McElroy and B. Glass (Baltimore: Johns Hopkins University Press, 1961), pp. 378–386.

[39] For example, a long wavelength form ($\sim$700 mμ) of chlorophyll-*a* probably acts as an energy sink for its own "accessory" pigments C_a 683 and C_a 695.[36] The energy difference here between the "sink" and the "accessory" pigments is sufficiently small that the "sinks" act only as very shallow traps for singlet excitation but probably as somewhat deeper traps[30] for triplet excitation. Efficient long-range transfer and annihilation effects at room temperature may occur between the sinks in both instances. The excitation, however, would be mostly localized near the sink pigments rather than spread equally throughout the photosynthetic unit, an advantage from a photochemical point of view. For the very shallow sinks which are presented to the singlet excitation, the room temperature fluorescence from the system is expected to originate primarily from the accessory pigments which are present in excess, while at low temperatures, the fluorescence should arise mostly from the energy traps. Certain recent experiments[40] can be interpreted in this way.

[40] Brody, S. S., *Science*, **126**, 838 (1958).

[41] *See*, for example, Tollin, G., E. Fujimori, and M. Calvin, these PROCEEDINGS, **44**, 1035 (1958). Near room temperature there persists a very long-lived fluorescence which presumably is caused by partial reversal of enzymatic steps.[42] This very slow component cannot be caused directly by triplet annihilation, but the shorter-lived components can be.

[42] Arthur, W. E., and B. L. Strehler, *Arch. Biochem. Biophys.*, **70**, 507 (1957); Strehler, B. L., in *Research in Photosynthesis: Proceedings of the Second Gatlinburg Conference on Photosynthesis of the National Research Council, October 25–29, 1955*, ed. H. Gaffron *et al.* (New York: Interscience Publishers, 1957), p. 118.

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*THE SPECIES VARIATION OF RNA METHYLASE**

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The presence of the methylated purines and pyrimidines in soluble RNA presents two questions: their origin and function. Assuming that all nucleic acid sequences are predetermined by the bases in DNA, the origin of nucleotide chains bearing methylated bases from a sequence of DNA which is devoid of them, appeared problematical. This anomaly was recently resolved by the demonstration of the existence in *E. coli* of an enzyme system (RNA methylase) which methylates s-RNA at the polynucleotide level[1, 2] using S-adenosyl-methionine as the methyl donor[3] for all bases, including thymine.[4]

A variety of functions has been ascribed to the methylated bases of s-RNA, ranging from their being mere disposal systems for surplus methyl groups to a pivotal role in the coding for amino acids. These hypotheses have been based on studies of the levels of the bases in the RNA of different species.

We have investigated not the quantitative levels of the various methylated bases, but rather the patterns of methylation by the RNA methylases from different species. From our studies we conclude that the methylation is highly ordered within a species and varied *inter* species.

The RNA from a given source, while fully methylated with respect to its homologous methylating enzymes, can offer new sites for methylation for the enzyme from a different species. In other words, there is a high reciprocal specificity between methylating enzymes and s-RNA substrate within each species. The implications of this finding on the possible role of the methyl groups in s-RNA will be discussed.

Experimental.—Preparation of homogenates containing the enzymes: Young rats, 6–8 weeks of age, were sacrificed by decapitation. The organs were quickly removed and homogenized in 4–6 volumes of ice-cold sucrose solution (0.25 *M*, 0.01 *M* $MgCl_2$) in a loose-fitting Potter-Elvehjem homogenizer. Nuclei, debris, whole cells, and mitochondria were removed by filtering through fine gauze and by centrifugation at 10,000 *g* for 15 min at 0°. The supernatant fluid was centrifuged at 105,000 *g* for one hr at 0°. The clear supernatant fluid was used for the enzyme studies.

TABLE 1

THE DISTRIBUTION OF RNA METHYLASE

Enzyme preparation	Methionine-methyl-C^{14} (cpm)		Adenosylmethionine-methyl-C^{14} (cpm)	
	Without RNA	With RNA*	Without RNA	With RNA*
Liver homogenate (1)	10	290	30	870
(2)	20	340	80	760
(3)	...	...	90	1,110
Brain homogenate	20	170	50	590
Spleen homogenate	30	580	110	1,840
Kidney homogenate	7	170	40	530
Heart homogenate	...	...	40	300
Spinach homogenate	10	70	150	1,600

* The nonmethylated s-RNA used was prepared from methionine-starved *E. coli* K_{12} W-6. For details see text.

The protein content of homogenates were: liver 16–20 mg; brain and heart, 7 mg; spleen 23 mg; spinach 8 mg per ml.

The extract of spinach was prepared by homogenization of 50 gm of fresh leaves and 100 gm of alumina (Alcoa, A-305) with 150 ml of K_2HPO_4 solution (0.1 *M*) in a Waring blendor for two min at 2°. The resulting homogenate was centrifuged at 20,000 *g* for 10 min at 0°. The supernatant fluid was again centrifuged at 105,000 *g* for one hr at 0°. The supernatant fluid from this centrifugation was assayed for its capacity to methylate different RNA preparations.

The reaction mixtures contained 100 μmoles of Tris buffer, pH 8.2, 100 μmoles of reduced glutathione, 100 μmoles of $MgCl_2$, 15 μmoles of enolpyruvylphosphate, 2 μmoles of ATP, 0.1 ml of a tenfold diluted crystalline preparation of pyruvate kinase (Boehringer and Soehne, Germany), either 0.6 μc of methionine-CH_3-C^{14} or 0.5 μc of adenosylmethionine, 1 ml of enzyme preparation plus additions in a total volume of 2.0 ml. The preparations of RNA, when added, were at a concentration of 2 mg in the total volume. The incubations were carried out at 37° for 45 min, at the end of which 1 ml of 3 *M* hydroxylamine (pH 7.4) was added. After 10 min, the tubes were cooled, 0.5 ml of 6 *N* HCl was added, followed by 1 ml of 20% TCA. The precipitate was collected by centrifugation and washed three times with 5 ml of 5% TCA containing a large excess of nonradioactive methionine or

adenosylmethionine. The residue was washed twice with 70% ethanol, followed by a wash with 3:1 ethanol ether mixture. The white precipitate was dried *in vacuo* at room temperature and the dry residue extracted with 2 *N* NH_4OH at 50°. The ammonia extract was transferred to stainless steel planchets, dried and counted in an end window counter.

The preparation of methylated and nonmethylated soluble RNA's from logarithmically growing organisms and methionine-starved organisms has been described earlier.[4] Soluble RNA from yeast was kindly provided by Dr. S. Morisawa; the soluble RNA and ribosomal RNA of liver were gifts of Dr. George Acs.

Results and Discussion.—In Table 1 the distribution of RNA methylase in various tissues is presented. In addition, its presence has also been demonstrated in white blood cells.[5] It is evidently a ubiquitously distributed enzyme. S-adenosylmethionine is invariably a more effective methyl donor than methionine plus ATP. This is probably a reflection of some limiting factor for the methionine-activating enzyme in our preparations.

The species specificity of RNA methylase was investigated by exposing various s-RNA preparations to the enzyme system from both a homologous and heterolo-

TABLE 2

INCORPORATION OF RADIOACTIVITY FROM ADENOSYLMETHIONINE-METHYL-C^{14} INTO DIFFERENT PREPARATIONS OF RNA BY HETEROLOGOUS ENZYMES

Additions	Enzyme preparation (cpm)	
	Liver homogenate	Spinach homogenate
None	50	130
Nonmethylated *E. coli* s-RNA	690	1,640
Methylated *E. coli* s-RNA	660	1,180
Yeast s-RNA	370	500
Liver s-RNA	100	...
Liver ribosomal RNA	140	140

For details see text.

gous source. An enzyme yields negligible incorporation of methyl groups into its homologous s-RNA. The s-RNA which accumulates during methionine starvation of *E. coli* K_{12} W-6 is an exception, since the RNA formed under these conditions is not methylated.[6] It should be emphasized, however, that at least one half of the s-RNA extracted from such sources contains the methylated bases, i.e., that fraction which had been synthesized under the normal conditions of growth prior to the methionine deprivation. It was found that the nonmethylated RNA accepts from the homologous enzyme system only its normal quota of methylated groups.[7a] Moreover, particulate RNA derived from ribosomes cannot serve as a methyl acceptor for the enzyme system. The sites for the methylation are thus highly specific (Table 2).

On the other hand, an enzyme apparently can search out new sites for methylation in an otherwise fully methylated heterologous s-RNA.[7b] It thus appears that both the substrate and the enzyme are endowed with a directive specificity which is a species characteristic.

These findings, although they do not clarify the problem of the function of methylation of s-RNA, do tend to exclude some of the putative roles for the process.

One of the superficially apparent functions of methyl groups is the reduction of hydrogen bonding sites which might contribute to the reversibility of attachment of

the adaptor area of the s-RNA to some template site. While this may have validity in the instances of substitution of nitrogen, it has recently been shown that the methylation of uracil to yield thymine has an apparently opposite effect: hydrogen bonding (by the carbonyl group of thymine) is increased.[8] Moreover, if the role of methylation were merely the alteration of hydrogen bonding, the species specificity and intraspecies variation of the enzyme system would appear to be superfluous. Nor is it likely in view of the findings reported here that the methylated bases are involved in the coding of amino acids for, if one assumes the amino acid code to be universal, the methylating enzymes of all species should be uniform. We find that a presumably encoded s-RNA of one species can accept methyl groups from a heterologous enzyme system.

The methylation of s-RNA does not appear to alter the susceptibility of s-RNA to nucleases for, at least with ribonuclease, we have found no appreciable difference in the rate of hydrolysis of the methylated and nonmethylated species of RNA.

The function of methylation is obscure and at the present time no satisfactory hypothesis can be offered for the role of the methyl groups in s-RNA.

It was suggested almost twenty years ago that chemical carcinogens alter transiently the structure of nucleic acids.[9] It was shown recently by Magee and Farber[10] that alkylating carcinogens alkylate s-RNA even more than they do DNA. Moreover, these authors find that the pattern of alkylation by chemical carcinogens is aberrant: sites in RNA which are normally free of methyl groups are alkylated; for example, 7-methyl guanine, a normally rare methylated base, abounds in the s-RNA of animals which had been exposed to methylating carcinogens.

Some of the attributes of the RNA methylase point to the possibility that under certain conditions this enzyme system may be a natural carcinogen.

The enzyme system is a complex of enzymes[3] with varied substrate specificities and the system has species variation. An oncogenic virus could introduce methylating enzymes foreign to the host. Such enzymes might then behave as alkylating carcinogens.

Bergquist and Matthews[11] have shown that some tumor tissues contain the highest levels of methylated bases in s-RNA ever reported. However, changes in the patterns of methylating enzymes of tumor tissue would be a more searching test of the hypothesis of RNA methylases as oncogenic agents. Such studies are in progress in our laboratory.

We are pleased to acknowledge the able assistance of Mrs. Louise Szybalsky in these experiments. We also wish to acknowledge gratefully the generous help of Drs. Thomas Staudt and H. Boyd Woodruff of Merck and Company, Rahway, N. J., for growing large quantities of bacteria for us.

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E. coli methylates s-RNA from liver but not its homologous methylated RNA. (Fleissner, E., to be published.)

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*RIBONUCLEIC ACID POLYMERASE OF AZOTOBACTER VINELANDII, II. FORMATION OF DNA-RNA HYBRIDS WITH SINGLE-STRANDED DNA AS PRIMER**

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RNA[1] polymerase, which catalyzes the DNA-dependent synthesis of RNA, is concerned with the transcription of the genetic message from DNA to RNA and is therefore a key enzyme in the expression of genetic information. For this reason the mode of action of the enzyme is of considerable interest. In producing complementary copies of primer DNA, the reaction might conceivably proceed by the intermediary formation of DNA-RNA hybrids. In contrast to free RNA such hybrids would be expected to be resistant to hydrolysis by a low concentration of RNAase.[2, 3] In the case of the reaction primed by native DNA, Geiduschek, Nakamoto, and Weiss[4] have concluded that stable hybrid intermediates do not accumulate. The finding, (by a number of workers) that when native primer is used, most of the RNA is sensitive to RNAase is consistent with this conclusion. As previously reported[5], heat-denatured, single-stranded DNA is a poorer primer of the enzyme than native DNA. On further examination it was found that the RNA synthesized with the former primer is markedly resistant to RNAase. Similar observations have recently been reported by Doerfler *et al.*[6] These results suggest that stable DNA-RNA hybrids are formed under these conditions. Closer investigation with the use of sucrose and Cs_2SO_4 gradient centrifugation, to be reported in this paper, has confirmed this suggestion.

The formation of stable DNA-RNA hybrids, when heat-denatured DNA is used to prime RNA synthesis, may explain the reduced effectiveness of single- versus double-stranded DNA and provides support for the idea that, in the normal operation of RNA polymerase, a single RNA strand is made on the double-stranded DNA template without separation of the individual strands.

Experimental Methods.—Materials and enzymatic assays: Labeled and unlabeled substrates, calf thymus DNA, and *Azotobacter* RNA polymerase were the same as previously described.[7] RNAase and DNAase were purchased from Worthington Biochemical Corp., Freehold, N. J. The enzymatic reaction mixtures were the same as those given under assay A of the previous paper using 20 μg of primer and 15 μg of RNA polymerase with a specific activity of 150 mμmoles C^{14}-AMP incorporated per mg of protein per min except where specifically noted. *Azotobacter vinelandii* DNA ($s_{20,w}$ = 22S) was prepared from freshly grown cells by modifications of the methods described by Marmur[8] and was further purified by precipitation with cetyltrimethylammonium bromide.[9] Heated preparations of DNA were made by exposure for 5 min at an ionic strength

TABLE 1

SYNTHESIS OF RNA WITH NATIVE AND HEAT-DENATURED DNA PRIMER AND SUSCEPTIBILITY OF PRODUCT TO RIBONUCLEASE

DNA primer	Acid-insoluble Radioactivity after Incubation* Control	Followed by RNAase†	Followed by heating ‡ and RNAase
Calf thymus, native	10,600	210	310
Calf thymus, heat-denatured	1,700	1,000	90
A. vinelandii, native	4,000	65	65
A. vinelandii, heat-denatured	825	490	103

* cpm/50 μl reaction mixture. Samples contained C^{14}-CTP (2.7×10^6 cpm/μmole) and nonlabeled ATP, GTP, and UTP. They were otherwise as previously described with addition of putrescine (final concentration, 0.04 *M*); 80 μg RNA polymerase (specific activity 60); 400 μg primer. Final volume, 1 ml. Incubation 15 min at 37°.

† 20 μg/ml for 30 min at 37° in a 10-fold dilution of the reaction mixture containing 0.002 *M* Mg^{++}.

‡ 10 min at 100° followed by rapid cooling and RNAase treatment.

of 0.015 to 90° for calf thymus DNA and 100° for *Azotobacter* DNA. No cross contamination of native DNA in the heated samples and vice versa could be detected by Cs_2SO_4 gradient centrifugation of a 30 μg sample.

Density gradient centrifugation: The Spinco analytical ultracentrifuge was equipped with a monochromator. Photographs were taken at a wavelength of 265 mμ. The gradients were established using Cs_2SO_4 at a density of 1.51 in a 1.0 cm column at 44,770 rpm at 25°. Cs_2SO_4 was purified by the method of Wake and Baldwin[10] and the density distribution in the cell at equilibrium was calculated employing their data. Solutions were made to the initial density by adding a weighed quantity of Cs_2SO_4 to the sample containing about 0.3 OD units measured at 260 mμ and adjusting the density by direct determination with a 0.3 ml pycnometer.

Other methods: Heating curves of absorbancy as a function of temperature were obtained as previously described.[5] G-50 Sephadex was obtained from Pharmacia Fine Chemicals, Inc., Rochester, Minn.

Results.—Susceptibility to RNAase of RNA synthesized with native or heated DNA as primer: As shown in Table 1 the RNA synthesized with native DNA (from either calf thymus or *A. vinelandii*) as primer was susceptible to hydrolysis with pancreatic RNAase. On the other hand, the product synthesized with heat-denatured, single-stranded primer was formed in lesser amount and showed considerable resistance to RNAase. As seen from the last column of the table, this product became RNAase-susceptible after heating, suggesting loss of secondary structure. In another series of experiments the incubated reaction mixtures were passed through a G-50 Sephadex column, previously equilibrated with 0.025 *M* Tris-HCl buffer, pH 7.6. This procedure was adopted in order to remove unreacted nucleotides and other compounds of low molecular weight. Under these conditions, the RNA synthesized in the presence of heat-denatured DNA as primer was found to be RNAase-sensitive. This result was due to the removal of Mg^{++} by the column since, after adjusting the effluent to 0.02 *M* Mg^{++}, the RNA was RNAase-resistant and, as in the experiments of Table 1, was rendered RNAase-sensitive by heating.

Density gradient centrifugation: Investigation of reaction mixtures using heat-denatured *A. vinelandii* DNA as primer, by sucrose density gradient centrifugation[11] in the Spinco preparative ultracentrifuge showed that while some of the acid-insoluble radioactivity sedimented slowly, a portion of the RNAase-resistant fraction sedimented at 22 *S* with the peak of nucleic acid and protein. This suggested the presence of a DNA-RNA hybrid species. However, the resolution of the zones in these gradients was poor and in further work Cs_2SO_4 gradients in the analytical ultracentrifuge were used. Sephadex column effluents as mentioned above, free of low molecular UV-absorbing material, were utilized for these experiments.

The results of a typical experiment, illustrated in Figure 1, show that the band corresponding to the heat-denatured DNA used as primer ($\rho = 1.451$) was completely missing and was replaced by a denser band ($\rho = 1.490$). The increased density of this band suggests that it corresponds to a hybrid species containing both DNA and RNA. Further evidence for this view was provided by examination of the Cs_2SO_4 banding patterns and acid-insoluble radioactivity of Sephadex column effluents after the following treatments. RNAase after Mg^{++} addition resulted in retention of the band of $\rho = 1.490$ while only a small portion of the radioactivity was rendered acid-soluble. After heating, the band was replaced by a band with the density of the single-stranded DNA primer ($\rho = 1.451$) while the acid-insoluble radioactivity was largely retained. The band was replaced by that of the DNA primer while the radioactivity was rendered almost completely acid-soluble after heating followed by RNAase treatment regardless of the presence or absence of Mg^{++}. The band observed in Sephadex effluents in the absence of Mg^{++} had the same density as in the presence of Mg^{++}, but disappeared completely after treatment with RNAase and was replaced by a band of the DNA primer.

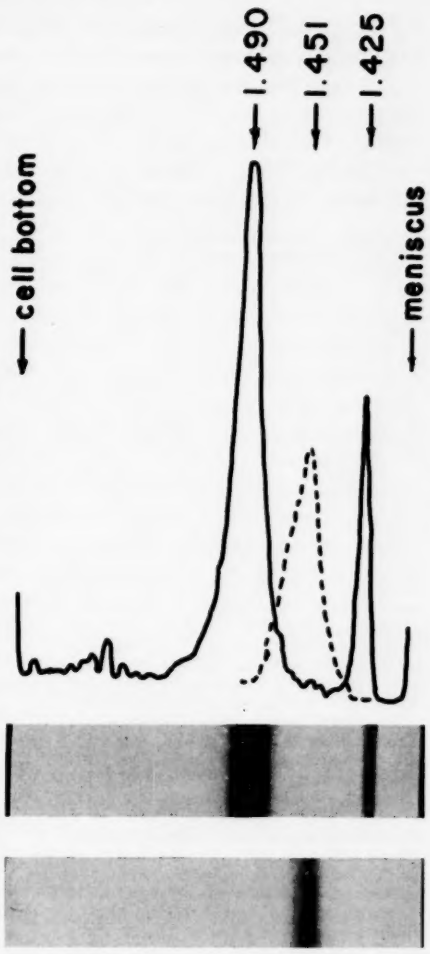

FIG. 1.—Banding of hybrids in a Cs_2SO_4 density gradient. Analytrol densitometer tracings are shown at the same linear magnification as the photographs of the bands. Upper photograph and solid line tracing show the hybrid band ($\rho = 1.490$) in a gradient in which native calf thymus DNA ($\rho = 1.425$) was used as a marker. This was obtained from a reaction mixture in which heated calf thymus DNA was used as the primer. The lower photograph and the dashed line tracing show a gradient containing only the heated DNA used as a primer in the reaction mixture from which the hybrid preparation was obtained. Mean density of Cs_2SO_4 in both cases was 1.51; column height in the cell, 1 cm; and rpm, 44,770.

It has not been possible to produce sharp bands in Cs_2SO_4 of the free RNA formed after heating the samples following incubation of labeled nucleoside triphosphates with RNA polymerase in the presence of heat-denatured DNA as primer, although an increase in optical density was evident near the bottom of the cell. Reaction mixtures primed with native DNA showed a much larger concentration of RNA near the bottom but still not sharply banded. The buoyant density estimated for the latter RNA was above 1.55. The poor banding of the

RNA is probably a result of its low molecular weight. On the basis of sucrose density gradient determinations, its sedimentation coefficient was found to be near 8 *S*.

The fact that the hybrid band was retained after treatment with RNAase, in the presence of Mg^{++}, whereas some of the radioactivity was rendered acid-soluble, suggests that although most of the RNA is present as a DNA-RNA hybrid, some may be free RNA. The relative quantities of DNA and RNA in the hybrid have not been precisely ascertained. The total synthesis of RNA, determined from the incorporation of radioactivity from C^{14}-labeled ribonucleoside triphosphates into acid-insoluble material, varied from 75 to 150% of the amount of primer used. Since some of this RNA is probably free, the hybrid may contain in general somewhat less RNA than DNA.

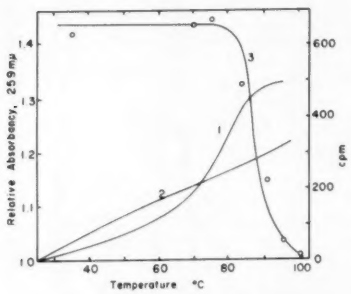

FIG. 2.—Temperature transition curves of hybrid. *Curve 1:* relative absorbancy at 259 mμ (left-hand ordinate) as a function of temperature. Measurements were carried out on Sephadex effluents (no Mg^{++} added) of a reaction mixture, with heat-denatured calf thymus DNA as primer, incubated under the conditions of the standard RNA polymerase assay. Heating in Tris-HCl buffer, pH 7.6; ionic strength, 0.1. *Curve 2:* same as curve 1 but with heat-denatured calf thymus DNA instead of Sephadex effluent. *Curve 3:* RNAase susceptibility of hybrid as a function of temperature. Measurements were carried out directly on aliquots of an incubated reaction mixture, similar to that of curve 1, with an estimated ionic strength of 0.15 and 0.02 *M* Mg^{++}. Each aliquot was exposed to the indicated temperature for 15 min and cooled rapidly. Acid-insoluble radioactivity was determined before and after treatment with RNAase as in Table 1. The right hand ordinate shows the radioactivity per 50 μl of reaction mixture in cpm that remained precipitable by acid after RNAase treatment. The acid-insoluble radioactivity before RNAase was 1,370 cpm.

Temperature-dependent transitions: The presence of an organized secondary structure in nucleic acids is most readily detected by the occurrence of a sharp transition from helical to random coil configuration as the temperature is raised. Changes in properties of the hybrid with temperature were investigated both spectrophotometrically and by determination of its susceptibility to RNAase. Curves demonstrating sharp transitions by both methods are shown in Figure 2. The transition temperature of about 77° observed in Sephadex effluents by the absorbancy change was about 10° higher in another experiment (not shown in the figure) carried out in the presence of added Mg^{++} at 0.02 *M* concentration. This is in agreement with the transition observed by RNAase sensitivity, an experiment which was conducted on the original incubation mixture containing 0.02 *M* Mg^{++}.

Discussion and Summary.—A DNA-RNA hybrid has been demonstrated to be formed during the synthesis of RNA by highly purified *A. vinelandii* RNA polymerase when the reaction is primed by heat-denatured, single-stranded DNA. The presence of both DNA and RNA in the hybrid is shown by several lines of evidence. (1) The buoyant density in Cs_2SO_4 is intermediate between that of DNA and RNA. (2) The resistance of the hybrid band to RNAase treatment is paralleled by similar resistance of the radioactivity from C^{14}-labeled ribonucleoside triphosphates incorporated into acid-insoluble material to the same treatment. (3) Heating the solution results in the disappearance of the hybrid band and the appearance of a band due to single-stranded DNA. The latter was demonstrated to be DNA by

the density of the band and by its disappearance on treatment with DNAase. Although there was no sharp banding of the released RNA, the acid-insoluble radioactivity became sensitive to RNAase indicating that RNA was released from a complex by heating. The sharp temperature transitions observed suggest a double-stranded structure for the hybrid.

Although heated DNA is a poorer primer than native DNA, net synthesis over the amount of primer added has been observed when using heated DNA. There are two possibilities that can account for this. (1) The single-stranded primer is used catalytically and the hybrid either dissociates to release free RNA or the RNA strand is displaced by the synthesis of another. (2) The hybrid, once formed, acts as a primer for further synthesis. The present data do not permit a distinction to be made between those possibilities. However, the stability of the hybrid and its accumulation in the reaction mixture give evidence that the strands of DNA are not separated in the reaction primed by native DNA. If strand separation occurred, one would expect hybrid of the type described here to accumulate in this case also. This is in agreement with the conclusions of Geiduschek *et al.*[4] In the absence of strand separation it is necessary that double-stranded primer be copied with equal probability from each end, producing an RNA strand complementary to one DNA strand in one direction and to the other DNA strand in the other. This is necessitated by the demonstration that the RNA formed has a similar nearest neighbor frequency to the DNA primer[12, 13] and by annealing experiments in which the RNA has been shown to contain strands complementary to each other[14] and to the primer DNA.[4] A possible mechanism for guiding such copying process has been suggested by Zubay in his model B.[15] *In vivo*, where the possibility exists that only one strand of the DNA is copied, there may be mechanisms for permitting the reaction to proceed in one direction only.

The accumulation of stable hybrid in the reaction primed by single-stranded DNA provides an explanation for the results of Wood and Berg.[16] These workers combined a protein-synthesizing system with RNA polymerase and found stimulation of amino acid incorporation except in the cases of heated T2 phage DNA and ϕX174 DNA. It is evident that most of the RNA synthesized by these single-stranded DNA's would be immobilized in the hybrid and would thus not be available to act as a messenger for amino acid incorporation.

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[1] Abbreviations: RNA, ribonucleic acid; DNA, deoxyribonucleic acid; RNAase, pancreatic ribonuclease; DNAase, pancreatic deoxyribonuclease; ATP, GTP, UTP, and CTP, 5′-triphosphates of adenosine, guanosine, uridine, and cytidine; Tris, tris (hydroxymethyl) amino methane; cpm, counts per minute.

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*DISTINCT CISTRONS FOR THE TWO RIBOSOMAL RNA COMPONENTS**

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Previous investigations[1, 2] have established that *E. coli* DNA contains sequences complementary to homologous ribosomal RNA. The proof depended on demonstrating the formation of specific RNAase resistant complexes between labeled ribosomal RNA and heat-denatured DNA. It was further shown, by the use of two identifying isotopic labels, that nonribosomal RNA from the same organism does not compete for the DNA sites complementary to ribosomal RNA.

All of these experiments were carried out with the 23S ribosomal RNA and they left unanswered the relation of these findings to the 16S RNA component. The similarity in base composition[3, 4] and the fact that the molecular weights[5] of the 23S and 16S are almost in the relation of 2:1 suggest the possibility of a common origin, the 23S being perhaps a dimer of the 16S RNA. Definitive evidence on whether they do, in fact, derive from the same sequence can be readily obtained with the hybridizing technique of Hall and Spiegelman[6] as modified in the ribosomal RNA investigations[1, 2] cited.

The following sorts of information are pertinent to a resolution. (a) *Saturation plateaus:* If the 16S and 23S are derived from the same sequence, the RNA/DNA ratio found in the hybrid at saturation should be the same for each RNA. (b) *Additivity:* At the saturation RNA/DNA ratio of either, the addition of the other should lead to no further complex formation if they are derived from the same sequences. If the sequences of origin are different, additional hybrids should be observed. (c) *Competitive interaction:* By the use of two identifying isotopic labels the presence or absence of competition during hybridization can be established. Absence of competitive interaction would indicate distinct sequences and its existence would argue for identity.

The present paper describes experiments which provide the data necessary for a decision. To alleviate somewhat the current monotony of molecular biology and to extend our understanding of these matters beyond *E. coli*, the experiments to be described were performed with *B. megaterium*. The results indicate that the sequences of the 23S and 16S RNA components are dissimilar. They must therefore possess different genetic origins.

Materials and Methods.—(a) *Bacterial strain:* Strain 219, a pyrimidine-requiring derivative of KM isolated by the technique of Mangalo and Wachsman[7, 8] was kindly provided by Dr. J. T. Wachsman.

(b) *Media:* A basal medium[7] supplemented with 10 to 30 μg/ml uridine was generally used. For P^{32} incorporation experiments, the phosphate concentration was reduced from 0.024 *M* to 0.0012 *M* and 0.05 *M* Tris (pH 7.3) added for buffering.

(c) *Preparation of cells:* Cells suspended in basal medium supplemented with 20–30 μg/ml in uridine were shaken overnight at 37°C, harvested, and resuspended at an $O.D._{660}$ of 0.200 in fresh medium containing 14 μg/ml uridine. When they attained an $O.D._{660}$ of 0.400, the cultures were harvested, washed, and resuspended in basal medium to an $O.D._{660}$ of about 1.000 for use as inocula in incorporation experiments.

(d) *Steady state isotope incorporation:* (1) *H^3-uridine:* Log phase cells, prepared as described, were suspended in basal medium to an $O.D._{660}$ of 0.035 and shaken at 37°C for 15 min. Then 10.3 μg/ml of H^3-uridine (New England Nuclear Corp., 3.0 mc/mM) was added and the culture shaken at 37°C until growth stopped at an $O.D._{660}$ of 0.240. The culture was harvested, washed, and resuspended in twice the original volume of basal medium containing 90 μg/ml of unlabeled uridine. The cells were incubated with aeration at 37°C for 0.8 generations to eliminate H^3 counts from the unstable RNA fraction, then harvested. (2) *P^{32}-orthophosphate:* Pyrophosphate-free, neutralized P^{32}-orthophosphate was added (630 μc/ml) to log phase cells at an $O.D._{660}$ of 0.07 in basal medium, containing 0.0012 *M* phosphate, and incorporation continued until an $O.D._{660}$ of 0.400 was reached. The culture was then "chased" for one generation in a medium adjusted to 0.024 *M* in nonradioactive phosphate.

(e) *Conversion to spheroplasts:* Log-phase cells were suspended to an $O.D._{660}$ of about 1.2 in a medium consisting of 0.04 *M* Tris, pH 7.3 − 0.002 *M* $MgSO_4$ − 0.3 *M* sucrose, and equilibrated to 37°C. Armour's lysozyme (200 μg/ml) was added and conversion to spheroplasts followed with a phase-contrast microscope. Conversion was virtually complete within 15 min. The spheroplasts were harvested, then washed once in the above medium.

(f) *Lysis and bulk RNA extraction:* Washed spheroplast pellets were lysed by resuspension in 0.01 *M* Tris, pH 7.3 − 0.005 *M* $MgCl_2$ (TM) buffer containing lysozyme (200 μg/ml) and 25 μg/ml of DNAase (Worthington Biochemical). The lysate was then subjected to three freeze-thaw cycles and total cellular RNA was isolated and purified, all as detailed by Hayashi and Spiegelman.[9]

(g) *Purification of ribosomal RNA subclasses:* The two ribosomal RNA components were separated from each other by repeated chromatography on methylated-albumin-kieselguhr (MAK) columns prepared according to Mandell and Hershey.[10] All buffers used during chromatography contained 0.025 *M* NaH_2PO_4 − 0.025 *M* Na_2HPO_4 − pH 6.9. RNA preparations were loaded at 50 μg/ml or less, and elution accomplished with linear NaCl gradients ranging from 0.6 *M* to 1.25 *M* NaCl. The total eluting volume was from 320 to 380 ml, and 5 to 7 ml fractions were collected. The resulting purified RNA fractions were pooled and concentrated to about 50 μg/ml as follows: the ionic strength of the solvent was first changed to 0.01 *M* Tris, pH 7.3 − 0.002 *M* $MgCl_2$ − 0.02 *M* NaCl by dialyzing against at least 100 volumes of this buffer for about 15 hr with two buffer changes. The preparations were next reduced to the appropriate volume in a flash-evaporator at reduced pressure. The sample flask was held at 28°C and the collecting flask at 0°C. Concentrated RNA preparations were finally dialyzed against TMS buffer (0.01 *M* Tris, pH 7.3 − 0.001 *M* $MgCl_2$ − 0.3 *M* NaCl).

(h) *Sucrose gradient analysis:* The size distribution of RNA preparations was routinely determined by centrifugation through linear sucrose density gradients.[9, 11]

(i) *DNA isolation:* DNA was extracted and purified from spheroplasts and heat-denatured as previously described for *E. coli*.[1] Heat-denatured preparations will be designated by 1XDNA.

(j) *DNA sedimentation velocity analysis:* Sedimentation coefficients were determined in the Spinco E analytical ultracentrifuge using UV optics. Runs were performed at 25 μg/ml according to the procedure of Marmur.[12] Observed sedimentation coefficients were corrected to 20°C. No other corrections were applied. Molecular weights were estimated from the measured S_{20} using the empirical relationship of Doty, McGill, and Rice.[13]

(k) *DNA-RNA hybridization:* All experiments described were performed with a heat-denatured DNA derived from a native preparation that had an S_{20} of 21.8 and an estimated molec-

ular weight of 7.3×10^6. DNA from *B. megaterium* undergoes renaturation rather readily; hence, the slow cool from higher temperatures employed in the earlier studies[1, 6] was avoided. Hybridizations were always performed by incubation at 41°–43°C.

Mixtures of 1XDNA at 50 μg/ml and labeled RNA at various concentrations in 0.7 ml of TMS buffer were incubated at 41°–43°C for 12 to 16 hr. Saturated CsCl was added to a final volume of about 3 ml and a density of 1.72. Centrifugation was carried out for 70 hr at 33,000 rpm in an SW 39 rotor of the Model L Spinco ultracentrifuge at a rotor temperature of 25°C. Fractions were collected from the bottom of the tube. Procedures for examining the DNA density region for RNAase resistant radioactivity on millipore membranes in a liquid scintillation spectrometer have been detailed by Yankofsky and Spiegelman.[1]

Results.—Purification of ribosomal RNA subclasses: Separation of labeled 16S and 23S RNA components was achieved by repeated chromatography on MAK columns. The purification was monitored by centrifugation in sucrose linear density gradients with unlabeled bulk RNA of *E. coli* added as size markers. The degree of cross contamination is readily determined by comparison of the radioactivity and O.D.$_{260}$ profiles.

An example of bulk *B. megaterium* RNA separation on a MAK column is shown in Figure 1. The profile is similar to those obtained in this laboratory with *E. coli* RNA preparations[14] except that the 16S region appears to be partially resolved into two components.

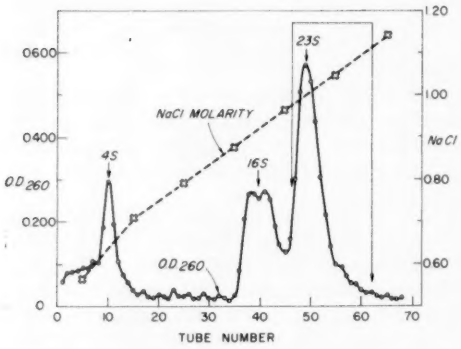

FIG. 1.—*Chromatographic separation of bulk RNA. B. megaterium* RNA was uniformly labeled with H^3-uridine as in *Methods*. The column was equilibrated at 0.66 *M* NaCl; the RNA loaded at 50 μg/ml in 0.66 *M* NaCl and eluted with a 360 ml linear gradient running from 0.66 *M* to 1.25 *M* NaCl. 5 ml fractions collected.

The 23S RNA region, indicated by the arrows in Figure 1, was chromatographed repeatedly, and the profile on the fourth column is shown in Figure 2*A*. Here, the arrows denote the region pooled and concentrated for experimental use, and Figure 2*B* shows its size distribution in a sucrose gradient. As can be seen, the purified labeled component is virtually confined to the 23S region of the carrier bulk RNA added.

The 16S component of Figure 1 was similarly treated, and Figure 3*A* shows a representative profile on MAK. Again, the arrows indicate the region pooled, concentrated, and analyzed for size. Figure 3*B* shows the size distribution of this region compared to that of *E. coli* marker RNA. Although of interest, the abnormality seen in the 16S profile both in Figure 1 and Figure 3*A* is not directly pertinent to the present investigation and its discussion will be deferred for a subsequent publication. Comparison of 3*A* and 3*B* indicates that the asymmetry observed is not due to significant contamination with 23S RNA. All preparations employed in the

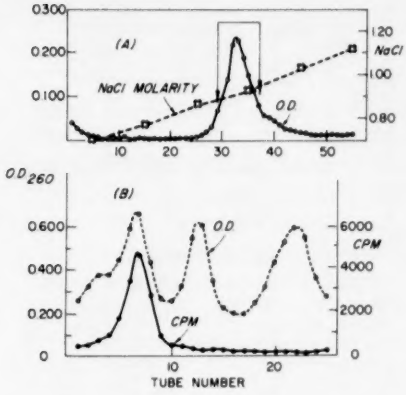

FIG. 2A.—*MAK column: Chromatographic profile after the fourth chromatography of the 23S region shown by the arrows in Figure 1.* The column was equilibrated at 0.68 *M* NaCl; 600 μg RNA loaded at 50 μg/ml in 0.68 *M* NaCl and eluted with a 320 ml linear gradient from 0.72 to 1.22 *M* NaCl.

FIG. 2B.—*Sucrose density gradient centrifugation.* An aliquot (0.5 μg RNA, 60,000 cpm) of the pooled tubes indicated by the arrows in (*A*) was used. 0.6 mg *E. coli* bulk RNA added as O.D. marker. 1.2 ml fractions collected and 0.3 ml samples from each tube plated for radioactive counts. The O.D. profile identified the known components in the added carrier material. The first major peak on the left is the 23S, the second the 16S, and the last corresponds to the 4S component.

present study were examined before use in sucrose gradients for cross contamination or evidence of breakdown. Samples showing evidence of either were discarded.

We now consider the details of the three types of experiments which can illuminate the origins of the 16S and 23S RNA components.

(1) *Saturation plateaus:* The proportion of RNAase resistant hybrid formed by incubating a fixed amount of 1XDNA with increasing amounts of each ribosomal RNA component are shown in Figure 4. The 23S RNA reaches a plateau when approximately 0.18% of the DNA is occupied, while about 0.14% of the DNA is capable of complexing with 16S RNA. In six repetitions, mean values of 0.179 ± 0.0072 and 0.136 ± 0.014 were obtained for the respective saturation values of 23S and 16S RNA. The fact that the saturation plateaus for the two are different supports the conclusion that the two types of RNA have different origins.

(2) *Additivity:* It will be noted from Figure 4 that for 50 μg DNA, saturation for the 23S RNA is achieved at 3 μg/ml, and 2 μg/ml saturates for the 16S component. We now inquire whether the addition of both at saturating levels to the same reaction mixture increases the amount of complex observed, and, if so, to what extent. The results of such an experiment are presented in Table 1. Addition of the values obtained when saturating amounts of each RNA subclass is complexed alone (mixture 1 + mixture 2) indicates that 0.303 per cent of the DNA would be hybridized. The amount of complex formed when both are incubated together (mixture 3) is within 4 per cent of this value. These results are difficult to reconcile

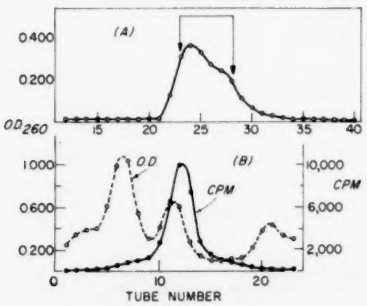

FIG. 3A.—*MAK column: Chromatographic profile of B. megaterium 16S RNA (steady state P^{32} label) after third chromatography.* Column equilibrated at 0.6 *M* NaCl; 760 μg RNA loaded at 50 μg/ml in 0.6 *M* NaCl and eluted with a 340 ml linear gradient from 0.6 *M* to 1.2 *M* NaCl.

FIG. 3B.—*Sucrose density gradient centrifugation: Analysis of an aliquot (0.5 μg of RNA, 80,000 cpm) from the pooled tubes shown under the arrows in* (*A*). 0.6 mg *E. coli* bulk RNA added as O.D. marker. All other details as in Figure 2*B*.

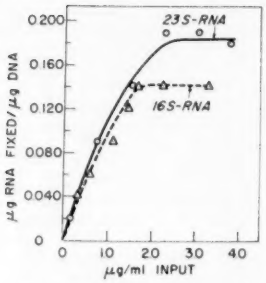

FIG. 4.—*Saturation plateaus: Saturation curves of B. megaterium 16S and 23S RNA hybridized with 50 μg/ml B. megateriumheat-denatured DNA.* Each point represents RNAase resistant counts found in the DNA region after CsCl equilibrium density gradient centrifugation. Annealing and analytical procedures as described under *Methods.*

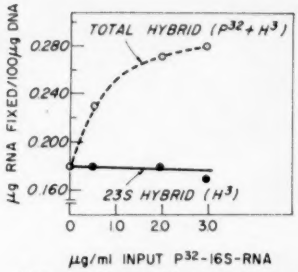

FIG. 5.—*Competitive interaction: Tests for competitive interactions between mixtures of 23S-H³-RNA and 16S-P³²-RNA from B. megaterium complexed with homologous heat-denatured DNA.* All mixtures contain 50 μg/ml DNA, 3 μg/ml purified 23S-H³-RNA (see Fig. 2) and the indicated concentrations of purified 16S-P³²-RNA (see Fig. 3). Annealing and analytical procedures as described under *Methods.*

with a common origin and are clearly consistent with the existence of distinct complementary regions. We come now to the final available experimental test.

(3) *Competitive interaction:* Increasing amounts of P³²-labeled 16S RNA were incubated in three tubes, each containing 50 μg of 1XDNA and H³-labeled 23S RNA at its saturation level (3.0 μg). Because of the identifying isotopic labels, it was possible to determine independently the per cent hybrid formed by each of the two ribosomal RNA size classes in the three mixtures. From the data shown in Figure 5 it is evident that the addition of the P³²-16S RNA results in no significant displacement of H³-23S RNA. Furthermore, as more 16S RNA is added, the total hybrid approaches a level of saturation near that expected for the sum of the two subclasses incubated alone. There is no evidence of competition between the two ribosomal RNA subclasses for common DNA sites.

Discussion.—In previous studies[1, 2] we have shown that specific complexes are formed between homologous ribosomal RNA and DNA in bacterial species having intermediate (52%) and high (64%) contents of guanosine-cytosine (GC) in their DNA. The present study establishes a similar sequence complementarity in *B. megaterium* which has a low (38%) GC content. The fact that ribosomal RNA

TABLE 1

TEST FOR ADDITIVITY DURING HYBRIDIZATION AT SATURATION LEVELS OF 16S AND 23S RNA

Mixture	Contents	RNAase resistant hybrid μg RNA fixed/100 μg DNA
1	50 μg/ml 1XDNA + 3.06 μg/ml 23S RNA	0.186
2	50 μg/ml 1XDNA + 2.21 μg/ml 16S RNA	0.117
		Sum = 0.303
3	50 μg/ml 1XDNA + 3.06 μg/ml 23S RNA + 2.21 μg/ml 16S RNA	0.291

The addition mixture (3) contained the same DNA and RNA preparations as the control mixtures (1 and 2). All three mixtures were annealed under identical conditions, centrifuged together, and the raw hybrids tested for RNAase resistance with the same enzyme preparation. Details of annealing and analytical procedures are given in *Methods.*

complementarity to DNA obtains in organisms of diverse DNA composition, lends credence to its generality.

These findings raise rather forcibly an interesting problem. The base composition of ribosomal RNA shows virtually no correlation with that of homologous DNA.[15, 16] Evidently the ribosomal RNA cistrons have been kept within narrow limits while the rest of the genome has undergone the widest variation in base composition permissible within a triplet coding mechanism. The specification of the selective mechanism which can produce this remarkable outcome poses an interesting problem for experimental resolution.

Sequence complementarity has previously been shown for the unstable messenger RNA,[9] transfer RNA,[17, 18] and 23S RNA.[1, 2] The present study demonstrates that it also holds for 16S RNA. Thus, the synthesis of all known cellular RNA components can be explained in terms of a DNA mediated reaction.

The present study had as its primary purpose to provide evidence which could decide whether the 16S and 23S ribosomal components are derived from the same or different complementary DNA sequences. The experiments reported indicate a difference in saturation plateaus, additivity of hybrid formation at saturation levels of each type, and absence of competitive interaction during hybrid formation. These findings are difficult to reconcile with a common sequence. They provide consistent evidence for distinct cistronic origins.

The further analysis into the nature of ribosomal RNA will require an examination for heterogeneity within each class. We have already pointed out[1, 2] that the level (0.2%) at which DNA is saturated by hybridizing with homologous 23S RNA would suggest that *E. coli* contains about 10 complementary cistrons for this component. The data presented here would suggest that 0.18% of *B. megaterium* DNA is complementary to its 23S ribosomal RNA and 0.14% to its 16S component. On the basis of the DNA content per "nuclear body"[19] one would estimate that the DNA contains approximately 35 stretches complementary to 23S RNA and 45 complementary to the 16S component. The significance of this apparent redundancy may be related to the rather large number of strands required for a full ribosomal complement which constitutes 85 per cent of the total cellular RNA. However, the existence of multiple copies in the genome provides a possibility for variation. It is of no little interest to determine whether this potentiality was exploited. It is evident that the use of column fractionation and competition experiments with identifying labels should provide data pertinent to this problem.

Summary.—The experiments reported were designed to decide whether the 16S and 23S ribosomal RNA components are derived from the same or different complementary sequences in the DNA. Specific hybrid formation, coupled with isotopic labeling was employed as the analytical device. The data establish that (*a*) the maximal amount of RNA which can hybridize per unit of DNA is different for the two; (*b*) at saturation concentrations of each, the amount of hybrid formed is additive when 16S and 23S RNA are both present; (*c*) no evidence of competitive interaction between the two for the same sites can be detected. All these findings are difficult to reconcile with a common origin. We conclude that 16S and 23S ribosomal RNA are derived from DNA sequences unique to each.

* This investigation was aided by grants-in-aid from the U.S. Public Health Service and the National Science Foundation.

† Predoctoral fellow trainee in Molecular Genetics (USPH 2G-319).

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*THE RELATIONSHIP BETWEEN RNA SYNTHESIS AND LOOP STRUCTURE IN LAMPBRUSH CHROMOSOMES**

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The experiments to be described are concerned with the relationship between structure and activity in the chromosomes of higher organisms, selecting, in particular, the giant "lampbrush" chromosomes of amphibian oöcytes. Ribonucleic acid synthesis in these chromosomes has already been shown by Gall and Callan[1] to occur on DNA-containing "loops" which project laterally from many sites along the chromosome axis. In the present paper, this chromosomal RNA synthesis is shown to be DNA-dependent, as judged by its sensitivity to Actinomycin D, and chromosomal structure is found to be intimately related to the capacity to synthesize RNA, as evidenced by changes in "loop" morphology induced by different actinomycins and by other agents, such as histones, which can also inhibit nuclear RNA synthesis.[2, 3]

Materials and Methods.—The "lampbrush" chromosomes which appear and increase in size in the course of meiosis seem especially well-suited for studies of chromosome function. At the diplotene stage, they remain enlarged and active for long periods while the oöcyte continues its maturation and growth. Lampbrush chromosomes of the newt, *Triturus viridescens*, were selected for study because of their great size and characteristic loop morphology, which facilitate observation and autoradiography, and because the intense synthetic activity of the nucleus at this stage in oöcyte development allows a measure of chromosome function based on the incorporation of radioactive amino acids into protein and of isotopically labeled purine and pyrimidine compounds into RNA.

For full accounts of the structure of lampbrush chromosomes, the reader is referred to E. B.

Wilson's text[4] and to more recent reviews,[5–7] especially to the superb memoir of Callan and Lloyd.[8] The essentials of structure relevant to these experiments can be summarized briefly as follows: at this stage in meiosis, each chromosome consists of two homologues of enormous length (400–700 μ[12]), joined at numerous bridges or chiasmata. A typical view is shown in Figure 1*A*. Each homologue has hundreds of loops which extend laterally, in pairs, from dense areas (centromeres) on the main chromosomal axis (Fig. 1*B*). These structural features are readily observable in the ordinary light microscope using freshly dissected, unfixed specimens (as shown by the pioneering work of Duryee[5]), but observations are much improved by the use of an inverted microscope with phase-optics (introduced by Gall[9]) and by employing the suspension media developed by Callan.[8]

The morphology of the loops at different sites differs sufficiently to allow their use for chromosome identification.[8] Each loop has a definite polarity, being thin where it leaves the main chromosomal axis and thick where it rejoins the centromere. Both the loops and the centromeres contain DNA, as indicated by their destruction upon exposure to deoxyribonuclease.[10] The loops also contain RNA.[11, 12] An important aspect of the structure is the inverse relationship between the length of the loops and the size of the centromeres from which they arise[8]; this is consistent with the view that the loops represent an uncoiling of the tightly wound chromatid at the centromere.

That loop formation is a sign of chromosomal activity is indicated by loop regression after the oöcyte reaches its full size, and by autoradiographic evidence that the majority of the loops rapidly incorporate H^3 uridine into ribonucleic acid.[1] The experiments now to be described indicate that the synthesis of RNA on the chromosome is mediated by a DNA "primer," presumably through the activity of an RNA-polymerase similar to those described in other cell types by Weiss,[13] Hurwitz *et al.*,[14] Stevens,[15] and Rho and Bonner.[16]

Oöcytes of the newt, *Triturus viridescens*, ranging in diameter from 0.9 to 1.2 mm were selected as corresponding to the "lampbrush" stage of chromosome development.[9] The chromosomes were isolated by microdissection as recommended by Gall[9, 1] and by Callan and Lloyd.[8] The nucleoplasm was dispersed in Callan's solution C[8] and the chromosomes were photographed under a Zeiss ("plankton") microscope with inverted optics.[1, 8, 9]

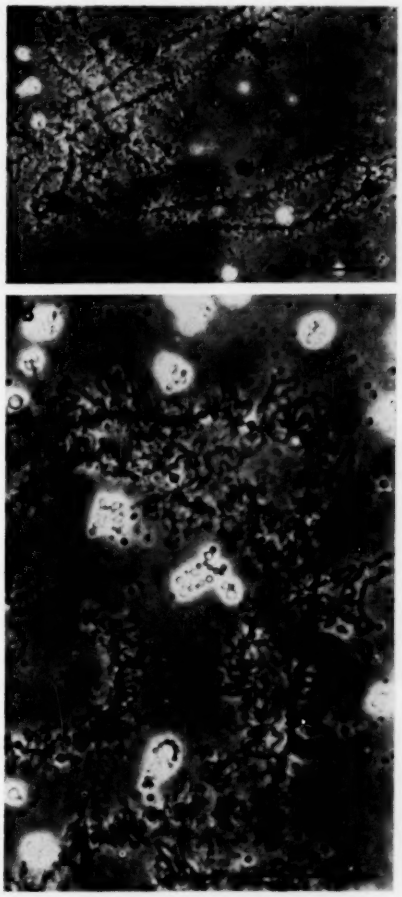

FIG. 1.—Lampbrush chromosomes of *Triturus viridescens*, unfixed: (*A*) low power view showing homologues joined at chiasmata, (*B*) higher magnification showing the loops projecting laterally from the main chromosomal axis.

The effects of the actinomycins on chromosome structure were determined using both intact oöcytes and isolated chromosomes. Actinomycin D and N-β-aminoethyl-Actinomycin C_3 were the generous gift of Dr. Edward Reich of the Rockefeller Institute. In whole egg experiments, these reagents were dissolved in 0.1 *M* NaCl-0.01 *M* "tris" (HCl) at pH 7.0 and the oöcytes exposed for periods ranging from 2 to 12 hr at 20°–22°C. After incubation the chromosomes were isolated and photographed. In testing the effects of the actinomycins on free chromosomes, the isolation was first carried out as described above,

and the chromosomes were then immersed in a small volume (0.25 ml) of actinomycin solution in a cylindrical well in a microscope slide. After periods ranging from 2 to 90 min, the chromosomes were photographed.

Similar techniques were applied in studying the effects of the histones and of polylysine on chromosome structure. The arginine-rich and lysine-rich histones were prepared from calf thymus by the method of Johns and Butler,[17] with the exception that we began with isolated nuclei rather than whole tissue.

In studies of RNA synthesis, H^3 uridine (0.9 c/mmol), H^3 adenosine (2 c/mmol) and H^3 cytidine (1.5 c/mmol) (Schwartz) were used as markers, and the incorporation was allowed to proceed in isolated oöcytes for 1.5, 3, and 6 hr at 20°–22°C. The chromosomes were subsequently isolated for autoradiography. When the actinomycins were tested, the eggs were preincubated for 5 hr at 20°–22° in 0.1 *M* NaCl-0.01 *M* "tris" at pH 7.0, containing 10 μg Actinomycin D (or N-β-aminoethyl Actinomycin C_3) per ml. After preincubation, the eggs were transferred to an Actinomycin-free medium containing 500 μc/ml H^3 uridine. Incubation was continued for 6 hr. Controls were preincubated in the saline-buffer without added actinomycin.

Localization of the isotope in RNA was tested using ribonuclease and deoxyribonuclease. After isolation of the radioactive chromosomes, some were immersed in 0.1 *M* NaCl-0.01 *M* "tris," pH 7.6, containing 0.4 mg/ml pancreatic ribonuclease (Worthington) and incubated for 2 hr at 37°. Other chromosomes were treated with deoxyribonuclease under the same conditions, or incubated in buffer alone. Following treatment, the chromosomes were prepared for autoradiography.

In studies of DNA synthesis, H^3 thymidine (6 c/mmol) was injected into whole animals. 300 μc, dissolved in 0.2 ml 0.1 *M* NaCl-0.01 *M* "tris," pH 7.0, was injected into the coelomic cavity. Five to fifteen days later, oöcytes were collected and the chromosomes prepared for autoradiography as described below.

Autoradiography of the isolated chromosomes following tritium-labeling experiments was carried out as recommended by Gall and Callan.[1]

Effect of Actinomycin D on Chromosomal RNA Synthesis.—In previous experiments on RNA synthesis in lampbrush chromosomes,[1] the isotopic precursor was injected into the whole animal. We have studied the process in isolated oöcytes, incubating them *in vitro* in the presence of tritiated uridine, cytidine, or adenosine, and then measuring the uptake in the isolated chromosomes by autoradiography.

These RNA precursors are readily incorporated in the *in vitro* system; increasing amounts of tritium are found in the chromosomes at 1.5, 3, and 6 hr incubation. Both the loops and the main chromosomal axis are labeled and grains are also observed over the nucleoli. In some chromosomal preparations, labeling is very intense along the main chromosomal axis, as shown in Figure 2. Virtually all of the isotope is removed from the loops and the axis by digestion with ribonuclease. DNase has no effect.

When Actinomycin D (Fig. 4*A*) is added to the suspension medium, the oöcytes lose the ability subsequently to incorporate tritiated uridine into RNA. Preexposure to Actinomycin D (at a concentration of 10 μg/ml) for 5 hr at 20° completely blocks RNA synthesis. No grains are seen over the chromosomes, nor is any labeling evident in the nucleoli.

Since Actinomycin D blocks RNA synthesis by selectively combining with the "primer" DNAs needed for the RNA polymerase reaction,[18–20] it can be concluded that virtually all RNA synthesis in the lampbrush chromosome is DNA-dependent. The failure to observe grains over the nucleoli as well indicates that nucleolar RNA synthesis is also DNA-dependent. These findings are in accord with earlier experiments on isolated thymus nuclei[21] and on mammalian cells in tissue culture.[18]

Effects of Different Actinomycins on Lampbrush Chromosome Structure.—The effects of Actinomycin D on chromosome structure were investigated using both in-

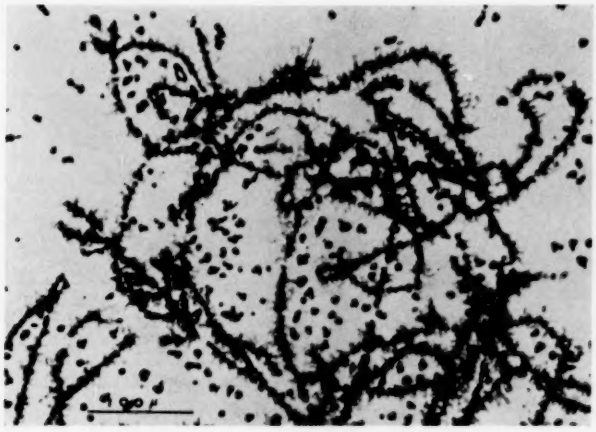

FIG. 2.—Autoradiograph of lampbrush chromosomes containing tritium-labeled RNA. The chromosomes were isolated from oöcytes previously incubated *in vitro* with H^3 uridine.

tact oöcytes and isolated chromosomes. Free oöcytes were exposed to 10 μg Actinomycin D per ml for periods ranging from 2 to 12 hr (an exposure sufficient to block RNA synthesis in the intact cell). The effects on chromosome morphology are striking; the characteristic loops disappear, although the main chromosomal axis remains intact. Presumably, the disappearance of the loops indicates their retraction into the chromosome.

The same effects can be seen in isolated chromosomes. With these, disappearance of the loops on exposure to Actinomycin D is very rapid; within 10 min the loops are gone from chromosomes immersed in Callan's Medium C[8] containing 2 μg/ml of the antibiotic. Compare the Actinomycin D-treated chromosomes in Figure 3*A* with the control chromosomes in Figure 1*B*.

Did this change in chromosomal morphology result simply from the combination between DNA and Actinomycin D, or did it follow as a result of the inhibition of RNA synthesis?

Experiments with a modified actinomycin support the latter interpretation. The compound used was N-β-aminoethyl Actinomycin C_3, which does not effectively inhibit RNA synthesis although it does combine with DNA.[22] Its structure

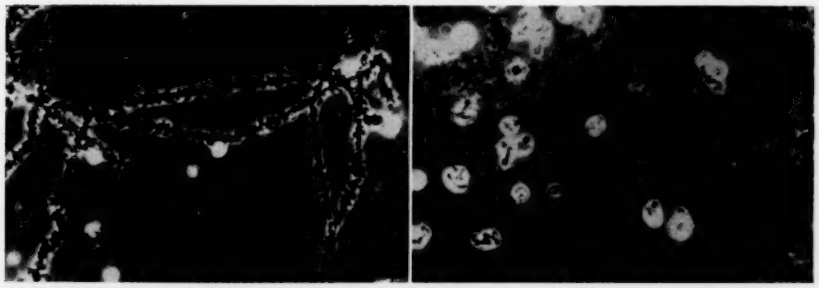

FIG. 3. —Effects of different actinomycins on chromosome structure. (*A*) View of lampbrush chromosomes after exposure to Actinomycin D (2 μg/ml) for 20 min at 16°C. Note disappearance of the loops. (*B*) View of chromosomes after 90 min incubation with 2 μg/ml N-β-aminoethyl-Actinomycin C_3. Note retention of the chromosomal loops.

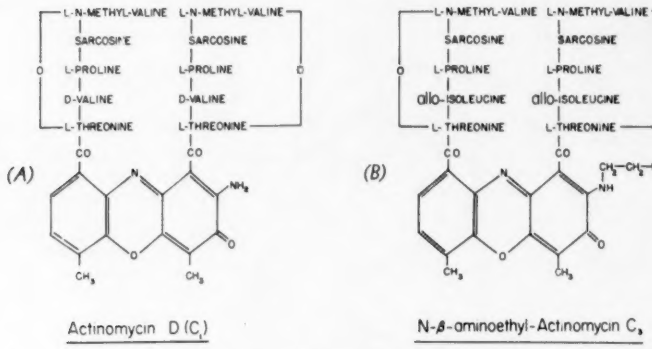

FIG. 4.—(*A*) Chemical formula of Actinomycin D. (*B*) Formula of N-β-aminoethyl-Actinomycin C_3.

is shown in Figure 4*B*. Lampbrush chromosomes exposed to 2 μg/ml of this "inactive" actinomycin did not lose their loops even after 1 or 2 hr incubation, nor was any effect seen at higher concentrations (10 μg/ml). Compare Figure 3*B* with Figure 3*A*.

In one experiment it could be shown that the modified actinomycin did combine with the chromosomal DNA because it protected isolated chromosomes against loop loss on subsequent attack by Actinomycin D. This protective effect is difficult to obtain regularly and has not yet been observed in intact oöcytes.

Since it is very likely that DNA binding by the modified Actinomycin C_3 does occur,[22] it is of interest that RNA synthesis is not inhibited and loop retraction did not occur. When Actinomycin D blocks RNA synthesis, the loops disappear.

The results strongly suggest that the existence of the loops is directly dependent on the continuing function of the chromosome, specifically in regard to RNA synthesis. This viewpoint is supported by three other lines of experimental evidence: (*a*) Loop formation does not depend on a continuing synthesis of DNA, since these chromosomes are not in the stage of DNA synthesis. (Thus, no incorporation of H^3 thymidine into lampbrush chromosomes has been observed, following injection of the whole animal with 300 μc of the DNA precursor, even after 5–15 days.) (*b*) The loops are not affected by other antibiotics, such as puromycin, which specifically block protein synthesis. (*c*) Other inhibitors of nuclear RNA synthesis, such as the arginine-rich histones, do cause immediate retraction of the loops. The latter two experiments are presented in more detail below.

Effects of Puromycin on Chromosomal Protein Synthesis.—Isolated *Triturus* oöcytes rapidly incorporate radioactive amino acids into their proteins, and the synthesis of proteins along the chromosomes is evident in autoradiographs. Figure 5*A* shows tritium labeling of the chromosomes after incubating whole cells for 6 hr at 20° with H^3 leucine. Grains are found over the loops and along the main chromosomal axis. As in other systems,[23] the uptake of radioactive amino acids into chromosomal proteins is effectively blocked by puromycin (50 μg/ml). Although protein synthesis in the loops is stopped by this antibiotic, puromycin does not destroy the loop structure as does Actinomycin D. Retention of the loops in puromycin-treated chromosomes (50 μg per ml for 30 min) is shown in Figure 5*B*. (Compare with

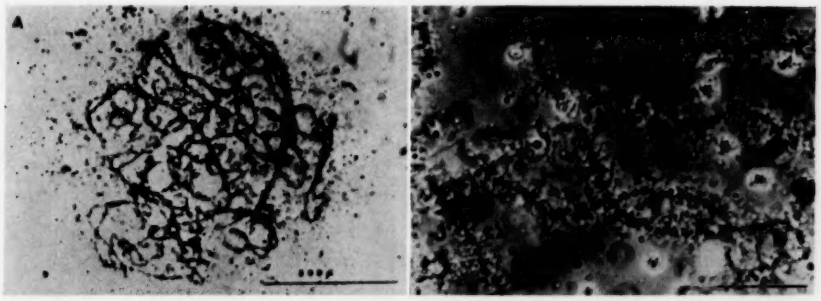

FIG. 5.—(*A*) Autoradiograph showing intense labeling of chromosomal proteins after 6-hr incubation of isolated oöcytes in the presence of H^3 leucine. (2.8 c/mmol; 500 μc per ml). (*B*) View of isolated lampbrush chromosomes exposed to puromycin hydrochloride (50 μg/ml) for 30 min at 16°C. Note retention of the loops, despite inhibition of protein synthesis.

the control chromosomes in Figure 1*B* and with Actinomycin D-treated chromosomes in Figure 3*A*.)

Effects of Different Histones and Polylysine on Chromosome Structure.—It has been shown previously that the addition of thymus histones to isolated nuclei inhibits RNA synthesis,[24, 2] and that histone-DNA complexes fail to act as "primers" for the RNA-polymerase reaction.[3]

The total histones of the thymus can be fractionated to yield arginine-rich and lysine-rich components.[17] We have shown that the arginine-rich histones are potent inhibitors of RNA synthesis, while the lysine-rich histones are weakly inhibitory or ineffectual.[2] For this reason, the effects of these different histone fractions were compared, observing changes in loop morphology in lampbrush chromosomes. As expected, the arginine-rich histones caused retraction of the loops; within 3 min all of the loops had disappeared from chromosomes exposed to 0.15 mg arginine-rich histone per ml (Fig. 6*A*). The lysine-rich fraction did not effect a comparable change in chromosome morphology. Even after 15 min, more than half of the loops remain on chromosomes exposed to 0.15 mg lysine-rich histone per ml (Fig. 6*B*).

In agreement with the earlier finding that polylysine itself is a potent inhibitor of nuclear RNA synthesis,[2] both poly-L-lysine and poly-D-lysine cause the loops to disappear from isolated chromosomes. Figure 6*C* shows the chromosomes after a 2 min exposure to 0.15 mg poly-L-lysine per ml.

Ribonuclease also causes the loops to disappear from unfixed chromosomes.

The complete correlation between the effects of these inhibitors of RNA synthesis on chromosome structure and the earlier results with the actinomycins strongly support the conclusion that *the morphology of an active chromosomal site is not only closely related to its functional role in RNA synthesis, but is dependent upon it.*

Although the lampbrush chromosome is an extreme example of chromosomal activity and development (for example, the RNA/DNA ratio is 10 or more, unlike most chromosomes where DNA predominates), one may suggest that the looping out of "primer" DNA, on a smaller scale, is a general phenomenon, and that chromosomal regions active in RNA synthesis may be "puffed" or extended, not only to allow their function, but because of it.

Summary.—Ribonucleic acid synthesis in lampbrush chromosomes occurs on loops

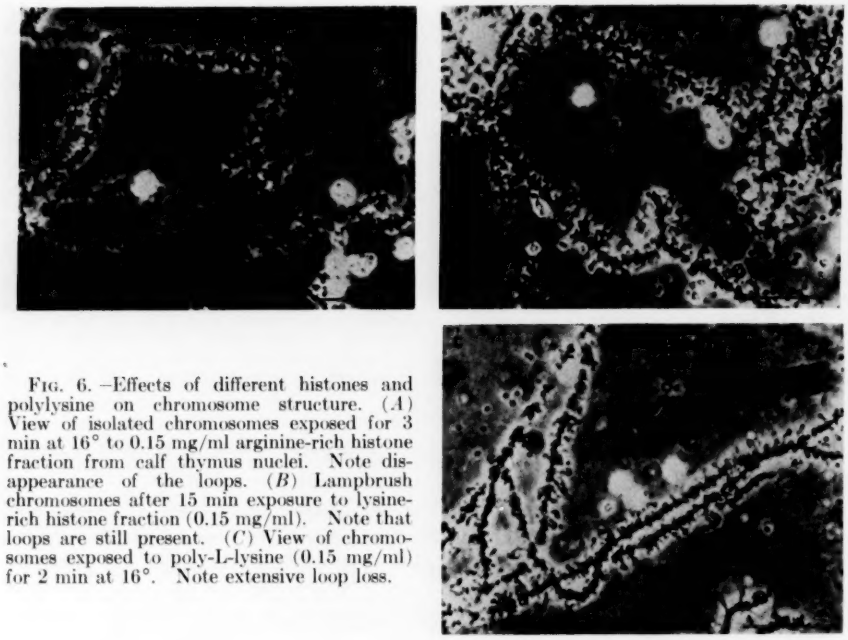

FIG. 6.—Effects of different histones and polylysine on chromosome structure. (*A*) View of isolated chromosomes exposed for 3 min at 16° to 0.15 mg/ml arginine-rich histone fraction from calf thymus nuclei. Note disappearance of the loops. (*B*) Lampbrush chromosomes after 15 min exposure to lysine-rich histone fraction (0.15 mg/ml). Note that loops are still present. (*C*) View of chromosomes exposed to poly-L-lysine (0.15 mg/ml) for 2 min at 16°. Note extensive loop loss.

which project from the main axis of the chromosome.[1] These loops contain DNA in addition to RNA.[10]

The synthesis of RNA in the chromosome is a DNA-dependent process, as judged by its sensitivity to inhibition by Actinomycin D. Nucleolar RNA synthesis is also blocked by this antibiotic. The addition of Actinomycin D to isolated chromosomes, or to intact oöcytes, not only inhibits RNA synthesis, but also leads to a disappearance of the chromosomal loops. A modified Actinomycin C_3 (which does not block RNA synthesis) does not cause loop retraction.

Other agents which inhibit nuclear RNA synthesis, such as the arginine-rich histones, also cause the loops to disappear. Inhibitors of protein synthesis, such as puromycin, do not have this effect.

The results suggest that the morphology of an active chromosomal site is not only closely related to its capacity to synthesize RNA, but is dependent upon it.

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*THE MOLECULAR SYNCHRONY AND SEQUENTIAL REPLICATION OF DNA IN ESCHERICHIA COLI**

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Cytological and genetic observations on a number of organisms have contributed to formulating the concept of a sequential replication of the chromosome starting from one or more fixed points.[1, 2] Especially in bacteria, as Maaløe[3] pointed out, this possibility found support in a mass of data strongly indicating a definite regularity in the synthesis of DNA.[4, 5] Advantage of the bacterial system was taken in the present work which employed two strains of *Escherichia coli* K-12 Hfr, as well as one of F^-, and analyses were made of the problem of molecular synchrony and the sequential replication of the chromosomes. A system has been established for determining the course of change in the number of prophage λ per bacterium during a replication cycle of DNA in populations of lysogenic bacteria under conditions of synchronous growth. A substantial amount of evidence indicates that the prophage λ is indeed a DNA structure, occupies a definite site on the K-12 linkage map, replicates in harmony with the host chromosome, and can be induced by various agents.[6–8] The existence of λ as an episome allows us to regard it as a gene on the bacterial chromosome and at the same time provides us with a means of enumerating its intracellular number at any given time. Thus, the kinetics of this measure should directly reflect the pattern of the replication of the host chromosome itself.

Bacterial Strains.—All the bacteria used are derivatives of *Escherichia coli* K-12 (Table 1).

Superinfecting phage: $\lambda v_1 h$, a weak virulent and host range mutant of λ,[9] was kindly provided by Dr. R. K. Appleyard. In contrast to the wild type (v_1^+) which forms turbid plaques, the mutant v_1 gives rise to clear plaques. High titer stocks of this phage were prepared by the method of confluent lysis.[10]

Media: (a) *M-9* is a minimal salt solution composed of 7.0 g anhydrous Na_2HPO_4, 3.0 g KH_2-

TABLE 1

BACTERIAL STRAINS

Strain	Remarks	Origin	Donor*
HfrH(λ)	m^-		M. Demerec
HfrCS101(λ)	m^-	58–161	M. Demerec
F⁻Z260(λ)	m^-	(F^+m^-)	N. D. Zinder
HfrH(λ, *424*)	m^-	HfrH(λ)	. . .
HfrCS101(λ, *424*)	m^-	HfrCS101(λ)	. .
F⁻Z260(λ, *424*)	m^-	F⁻Z260(λ)	. . .
C600	$t^-l^-B_1^-$, sensitive to λ & *424*	679–680	R. K. Appleyard
C600(λ)	$t^-l^-B_1^-$, sensitive to *424*	C600	. . .
C600(*424*)	$t^-l^-B_1^-$, sensitive to λ	C600	F. Jacob

The symbols designate as follows: *m*, methionine; *t*, threonine; *l*, leucine; B_1, Vitamin B_1; (λ), lysogenic for λ; (*424*), lysogenic for *424*. The double lysogens for λ and *424* were isolated after infecting each λ lysogen with the temperate phage *424*; C600(λ), likewise after infecting C600 with λ.

* The author is greatly indebted to these donors for their generosity.

PO_4, 1.0 g NH_4Cl and 1 liter of distilled water. After autoclaving, a separately autoclaved stock solution of $MgSO_4 \cdot 7H_2O$ is added to a final concentration of 5×10^{-4} *M*, the pH being 6.8–7.0.

(b) *M-9(0.05):* To the *M-9*, glucose and DL-methionine are added to final concentrations of 0.05% and 50 μg/ml, respectively. It is used to grow tube cultures and gives a final yield of bacteria of 5×10^8 cells/ml.

(c) *M-9(0.1)* is same as *M-9(0.05)*, except that the final concentration of glucose is 0.1%; it is used to grow flask cultures and gives a final yield of bacteria of 10^9 cells/ml.

(d) *YTB* is a growth medium for indicator bacteria as well as for postinduction incubation, and is composed of 5 g Yeast Extract (Difco), 10 g Bacto-Tryptone (Difco), 5 g NaCl and 10 ml of a 25% $MgSO_4 \cdot 7H_2O$ solution, dissolved in 1 liter of distilled water.

(e) *YTA:* To the *YTB*, 1.7% Bacto-Agar (Difco) is added.

(f) *Soft agar:* Same as *YTA* except that the agar content is 0.7%. It is used to plate phage particles for plaque counting by the agar layer technique.[11]

Methods: (a) *Synchronization of bacterial growth* was achieved by employing a fractional filtration technique originally described by Maruyama and Yanagita;[12] several important modifications were made, however. In order to start each experiment with a bacterial population as homogeneous as possible, a single colony was isolated and grown on a fresh *YTA* slant for about 6–8 hr at 37°. From this young clone, a small number of cells was inoculated into 5 ml *M-9(0.05)* for overnight incubation with aeration at 37°. After washing the overnight culture twice, cells were inoculated into 300 ml of fresh *M-9(0.1)*. The culture was incubated at 37° on a shaker for more than 10 hr whereupon it reached a stationary phase; thereafter, incubation was continued for 120 more min. This timing is important in that a relatively homogeneous and healthy population of cells largely determines success in synchronization. After centrifugation of the culture, cells were washed once and recovered as *ca.* 1.4-1.7 ml of thick suspension which was ready for filtration. A filter paper pile was prepared with 18 sheets of No. 126 Toyo filter paper sandwiched between 2 sheets of No. 1 Toyo filter paper at the top and 1 sheet at the bottom. The pile was tied with a piece of string, placed in a funnel, and an inner cylinder was screwed above it. The pile must be moistened with 3 ml of prewarmed *M-9* for 10 to 15 min before use. The thick suspension of cells, prepared as above, was first adsorbed on the top sheets of the pile. This was achieved within 2 min, whereupon the inner cylinder was screwed tightly and 10 ml of prewarmed *M-9* was added; simultaneously the suction decompression was applied. Two or three successive elutions with 10 ml *M-9* were found necessary in order to obtain a good recovery of cells in the filtrate (usually 10–20%). The entire process of elution should take only about 1 min. The filtrate was centrifuged, an appropriate amount of the pellet was inoculated into 300 ml of fresh prewarmed *M-9(0.1)*, and incubation in a 37° shaker-waterbath followed immediately.

(b) *Measurement of DNA net synthesis* was carried out by taking samples at every 10 min during the postfiltration incubation; the samples were subjected to the method described by Burton.[13]

(c) *The absolute number of prophage* λ *per bacterium* was determined by the technique[14] which involves induction of λ and superinfection with $\lambda v_1 h$. The Poisson distribution was introduced

as a correction factor in order to make possible the analysis of the lysate of a population instead of just single bursts.[15] Every 5 min in the postfiltration incubation 6 ml samples were withdrawn and irradiated with UV for 20 sec with a Westinghouse sterilamp (46 erg/sec/mm^2). Four ml of the irradiated suspension were inoculated into 1 ml of 5 × *YTB* where a suitable number (depending upon the multiplicity desired) of superinfecting phage $\lambda v_1 h$ was present. Incubation followed immediately in a 37° waterbath with aeration by bubbling. After 15 min of post-ultraviolet incubation, a 0.1 ml sample was taken to determine the number of unadsorbed $\lambda v_1 h$. After 130 min, the lysate was chloroformed; the supernatant was then plated on very thick *YTA* (not less than 1 cm) with a growing culture of *E. coli* K-12 C600.

(d) *Determination of the relative number of two prophates, λ and 424:* In order to avoid the ambiguity caused by the superinfection technique which requires some correction procedures, the system was improved by eliminating superinfection and introducing a second prophage, *424*, as a reference for λ. All the strains, already lysogenic for λ and used before, were further lysogenized with *424*. The resulting double lysogens were subjected to exactly the same procedures for synchronization, viable counts, and DNA determination. For prophage enumeration, samples were simply induced by UV; the titers of phage λ and *424* in the resulting lysate were determined by plating the supernatant on *YTA* with the appropriate indicator bacteria. These were *E. coli* strains K-12 C600 (*424*) for λ and C600(λ) for *424* (because of their immunity differences); their plating efficiencies were not significantly different.

Results.—Kinetics of prophage duplication during one cycle of DNA replication: After precautions in every step of the procedure for synchronizing cell division, all strains responded by a satisfactory stepwise growth. DNA increased exponentially through several generations with a probable break at each onset of cell division; thus, one DNA replication cycle corresponded almost exactly to one generation of cell division. This roughly agrees with the finding with nonsynchronized populations that DNA synthesis occupies more than 80 per cent of a cell generation.[4] The kinetics of increase in turbidity also roughly corresponded with that of DNA.

(a) *Change in the absolute number of prophage λ per bacterium:* In all the samples for prophage determination, taken at different times during the cell growth and infected with various multiplicities of $\lambda v_1 h$ (*ca.* 2–10), practically all the cells in a population were lysed. The average burst size increased linearly from about 80 to 120 as a function of time within one replication cycle. No significant difference was observed in the burst size obtained after induction alone and after induction plus superinfection. It was proved in a system equivalent to the present one that both genotypes of λ, v_1^+ and v_1, are not subject to differential selective forces during their vegetative multiplication together.[16] Therefore, the ratio of the number of turbid plaques to the number of clear ones, obtained by plating the lysate, can be taken as a direct function of both the multiplicity of superinfecting phage and the number of intracellular prophage at the time of induction and superinfection; the last parameter can be calculated by knowing the first two.[15]

Figure 1*a* shows the pattern of prophage kinetics in HfrH(λ). The number of prophage per bacterium remained at a constant value until the net increase of DNA reached approximately the 50 per cent level. Then an abrupt doubling in prophage number was achieved at a time when the increase in total DNA was 80 per cent; toward the beginning of the next replication cycle the number returned sharply to the initial value. In another strain of Hfr, CS101 (λ), however, the number of prophage doubled quickly during a very short period after the beginning of the replication cycle, remained constant throughout the rest of the cycle until the onset of the next (Fig. 1*b*). At the time of the doubling in prophage number, the DNA increase was only at a level of *ca.* 10 per cent. The F$^-$(λ) displayed yet

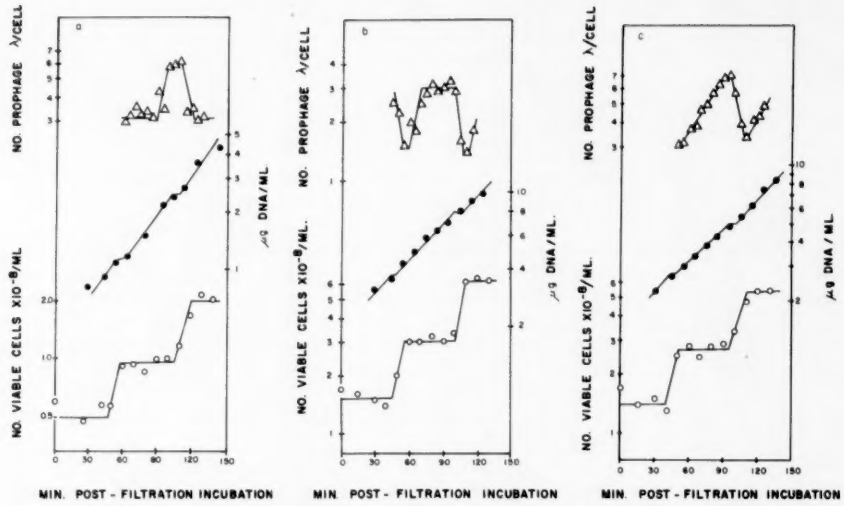

FIG. 1 *a–c.*—Change in the absolute number of prophage λ per bacterium over one replication cycle of DNA in synchronized populations of *E. coli* K-12. The intracellular number of λ(Δ) was determined by the induction-superinfection technique described in *Methods*. Synchronization was achieved by fractional filtration; DNA (●) was determined by the Burton-Dische method; the number of viable cells (○), by plating samples on agar plates. (*a*) HfrH(λ) with average multiplicities of superinfection, *n*, of 4.8–8.9. (*b*) HfrCS101(λ) with $n = 6.4–11.3$. (*c*) F⁻Z260(λ) with $n = 2.8–4.8$.

another pattern of prophage kinetics (Fig. 1*c*) where no abrupt doubling of the number was observed. When cells were grown nonsynchronously, these specific prophage patterns were completely distorted to the extent that the number of prophage per cell did not significantly change. It can be concluded, therefore, that the observed difference in the prophage kinetics is significant; each of the unique patterns is characteristic of each strain of bacteria, attributable only to the synchronous growth of the cells, and hence reflects the molecular synchrony of DNA itself.

(b) *Change in the relative number of prophages, λ and 424, per bacterium:* Upon induction of bacteria doubly lysogenic for λ and *424*, both prophages gave rise to progeny phages in the lysate. The average burst size was not significantly different among the different strains; it was also the same for single lysogens carrying either of the prophages as for double lysogens, growing synchronously or not. The same type of consistency was also observed for the per cent induction of λ (more than 96%) and *424* ($25.7 \pm 1.4\%$). DNA synthesis stops immediately after irradiation with the inducing dosage of UV and is arrested for about 30 min during post-UV incubation. All these facts justify taking the ratio of λ/*424* in a lysate as a measure of the intracellular ratio of the two prophages at the moment of sampling and induction.

In HfrH(λ, *424*) (Fig. 2*a*), the ratio stayed at a constant value nearly halfway through the course of increase of DNA, whereupon it was sharply reduced to almost one half. Then it resumed the initial value at a time when the DNA had increased about 80 per cent. On the contrary, the ratio in HfrCS101 (λ, *424*) (Fig.

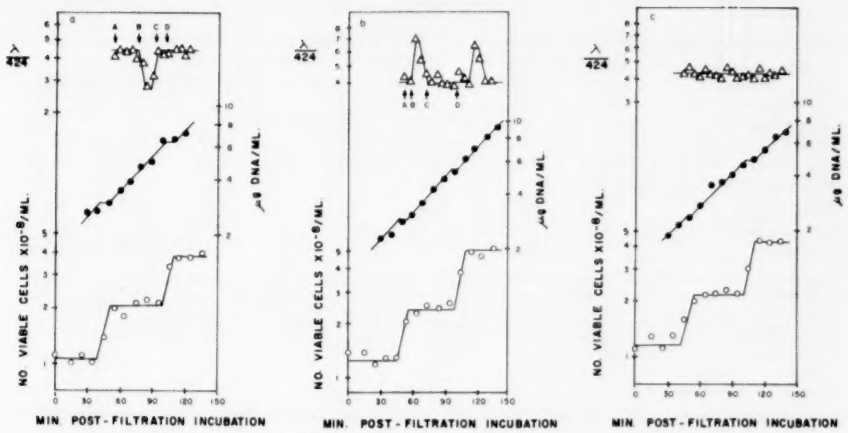

FIG. 2 *a–c*.—Change in the relative number of prophages λ and *424* per bacterium during one replication cycle of DNA in synchronized populations of *E. coli* K-12. The relative number was expressed in terms of the ratio (Δ) of phage λ to phage *424* in the lysate obtained after induction of double lysogens with UV. All the conditions for synchronization, DNA (●) determinations and viable counts (○), are similar to those described for Figure 1. (*a*) HfrH(λ, *424*); the sharp decrease in the ratio is significant ($P \ll 0.01$) by *t* test. (*b*) HfrCS101(λ, *424*); the sharp increase in the ratio is significant ($P \ll 0.01$) by *t* test. (*c*) F⁻Z260(λ, *424*); the ratios scattered around a mean of 4.35 ± 0.11.

2*b*) doubled almost immediately at the beginning of synchronous growth, returned to the original level when the DNA had increased about 40 per cent, and thereafter remained constant until the beginning of the next generation. In F⁻(λ, *424*) (Fig. 2*c*), the ratio remained constant throughout the cycle. These observations are all consistent with the previous findings and distinguish even more clearly the relative time of duplication of the prophages in each Hfr strain.

Polarity in the replication of bacterial chromosomes: It is known that the point of rupture on the continuous circular chromosome of *E. coli* K-12, the location of the F factor on the resulting linear structure, and hence the direction of genetic transfer by each chromosome at mating are characteristic of each Hfr strain.[7, 17] The relative positions of λ and *424* on such a linkage map of each Hfr were compared with their relative time of duplication expressed in terms of per cent increase of DNA with a marked agreement between the two sets of values (Table 2). Moreover, the fact that the timing of λ duplication in Hfr strains determined by two different methods was proved to be consistent suggests the validity of the observations. All these results lead to the conclusion that the replication of the Hfr chromosome is synchronized and has a definite polarity starting from its posterior end where the F factor is attached and proceeds to the forward end. There is an assumption here, however, that the map distance of genetic loci including prophages, expressed in terms of the time of entry upon conjugation, designates their geographical positions on the actual DNA molecule; this may be the case, especially in view of P^{32} decay experiments where it was shown that the kinetics of genetic transfer at conjugation could be correlated with the kinetics of nucleotide transfer.[7, 19]

The prophage kinetics of F⁻ (Fig. 1*c*) shows perfect harmony with the mutation kinetics of *E. coli* 15 (F⁻) thymineless bacteria induced with 5-bromouracil during

TABLE 2

COMPARISON OF THE RELATIVE POSITIONS OF TWO PROPHAGES AS DETERMINED BY TIME OF DUPLICATION WITH THEIR POSITIONS ON THE LINKAGE MAP

Strain	HfrH (λ, *424*)				HfrCS101 (λ, *424*)			
Points on Fig. 2*a*, *b*	A	B	C	D	A	B	C	D
μgDNA/ml	2.9	4.4	5.4	6.0	2.9	3.1	4.0	5.9
Δ DNA to point A	0.0	1.5	2.5	3.1	0.0	0.2	1.1	3.0
% increase of DNA	0.0	48.3	80.5	100.0	0.0	6.7	36.7	100.0
Markers on the linkage map	F	*424*	λ	*t*	F	λ	*424*	*lac*
Distance from F	0′	57′	92′	108′	0′	6′	41′	108′
% distance	0.0	52.8	85.0	100.0	0.0	5.5	38.0	100.0

Point A represents the start of a generation cycle; B, the time of the first prophage duplication; C, that of the second prophage duplication; and D, the end of the cycle. μgDNA/ml for each point were read from the DNA curves in Fig. 2*a* and *b*. Markers on the linkage map: F designates the F factor attached to the tail end of the chroromosome; *424* and λ, the prophages; *t*, threonine; and *lac*, lactose. The last two genes occupy positions at the head ends of the chromosomes.[7, 17] The distance of each marker from F is expressed in terms of time of entry upon conjugation in minutes.[18]

a synchronous growth caused by thymine starvation.[20] Recent autoradiographic studies[2] have revealed that even the replication patterns of the chromosomes are quite similar in F⁻ and Hfr. These facts seem to indicate that the F⁻ chromosome replicates also with polarity but starts at random from any site on the continuous circular structure. Accordingly, if one used thymineless Hfr for the mutation study, a pattern of induction kinetics similar to that of prophage should be expected; some preliminary results are at hand suggesting this is indeed the case in such a system where a mutagenic base analogue was pulsed into the synchronously replicating DNA.[21]

Discussion.—Although the possibility of a "conservative" replication of DNA *in vivo* has not yet been completely ruled out,[22] evidences have been accumulated which indicate that the replication is "semiconservative"; this was most clearly shown in bacteria,[23] where "side-to-side" hybrids were formed rather than "end-to-end" association of subunits.[24] A question arises, however, as to whether or not this mechanism also characterizes the replication of DNA molecules at higher levels of organization, e.g., in the whole intact bacterial chromosome. The autoradiographic studies of Cairns[2] are illuminating in this regard, and his conclusion was that the long molecule of DNA (more than 700 μ) is double-stranded, and replicates from one fixed point in a semiconservative fashion. These results are readily compatible with the present prophage data.

Based on the very likely assumption that the long molecule of DNA also undergoes semiconservative replication, an attempt was made to construct molecular models (Fig. 3) of chromosome replication in order to account for the prophage data. In Model I, one expects λ to duplicate when DNA replication is 85 per cent complete in HfrH and 5.5 per cent in HfrCS101, whereas the observed values are 80 per cent and 6.7 per cent, respectively (cf. Table 2), showing a marked agreement. In view of studies on the *in vitro* synthesis of DNA,[25] however, one might argue that replication starts from both ends of the molecule with unwinding of the double helix, leaving each of the partner strands temporarily single.[26] Although it was argued that this need not necessarily apply,[27] the possibility still remains also at the chromosomal level and was examined in Model II. If we assume that the temporarily single-stranded molecule of λ DNA, attached to the host molecule in a yet unknown way, can be induced, as well as the newly formed double-stranded λ, to

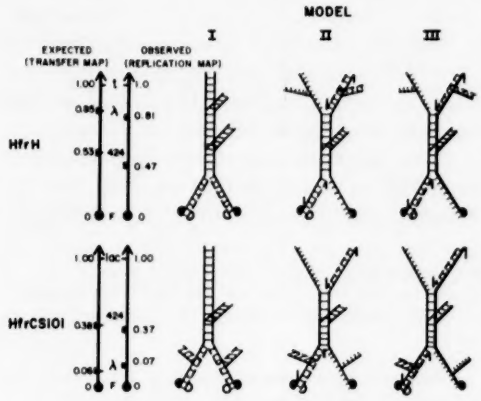

FIG. 3.—Molecular models for the sequential replication of Hfr chromosomes. The solid lines in these models indicate the parental DNA strands and the broken lines, new growing strands. The thick lines in Model III indicate the particular strands assumed to carry information for responding to UV induction to produce normal phage progeny (see text). Arrows in the Models II and III show the antiparallel polarity in the backbones of the double-stranded DNA determined by the phosphodiester bonds of the deoxyribonucleosides. Although the molecular nature of the attachment of the prophages to the host chromosome is not known, the prophages are indicated as branches to make the illustrations clear.

give rise to healthy progeny phages, the expectation would be that λ prophage duplicates upon a 5.5 per cent increase in DNA in CS101, in agreement with the observation (6.7%). But λ should double when DNA has increased by 15 per cent in HfrH, in disagreement with the observation (80%); the two observations are evidently contradictory. If we assume otherwise, that single-stranded λ cannot be induced, then the expected value for HfrH (85%) agrees with observed (80%); but this is not the case for CS101, expected (94.5%) versus observed (6.7%). Again there is an obvious contradiction, indicating that the model is invalid. Yet another possibility could be entertained; namely, if we assume that only one of the two strands carries information for responding to the inducing agent, then Model III can be formulated. Suppose only the upward strand in each strain of Hfr carries the information, and replication takes place in a manner similar to Model II. Suppose further that the new chain growing upward from the end where F factor is located also carries the information, then this particular chain is the one which decides the timing of λ duplication as detected by induction. This model is as compatible with the prophage data as Model I and cannot be ruled out by the prophage technique alone. Against this hypothesis, however, is evidence that both members of a base pair must be changed upon mutation for a mutant phenotype to be expressed.[28] Furthermore, the Kornberg enzyme could still operate in the way postulated in Model I, if the bacterial chromosome consisted of a large number of subunits, each of which replicated in an antiparallel fashion. The results of Cairns also support Model I. Here the most challenging question concerns the nature of the controlling mechanism that determines the replication polarity; what factor plays the role of primary importance—the DNA polymerase, hydrogen bond formation, unwinding itself, or some unknown factor?

Summary.—The question of the mechanism of chromosome replication in bacteria has been studied with lysogenic strains of *Escherichia coli* K-12 Hfr and F^-. Bacterial growth was synchronized by the fractional filtration technique, the smaller

cells being recovered. One replication cycle of DNA corresponded to a synchronized cell generation. Kinetic studies of prophage duplication over one replication cycle of DNA revealed: first, that the pattern of kinetics is distinctively strain specific; second, that this is brought about only by synchronization; and finally, that the specific time of prophage duplication, expressed in terms of per cent increase of DNA, can be directly correlated with the known genetic linkage map.

These findings led to the conclusion that the replication of the chromosome in *E. coli* K-12 is synchronized and sequential, especially in Hfr, starting from the posterior end where the F factor is attached and proceeding to the forward end.

The author should like to express his sincere gratitude to Professor Francis J. Ryan for his patient guidance, constant encouragement, and interest throughout the course of this investigation, to Professors J. H. Taylor, J. Cairns, E. E. Clark, and to Drs. R. K. Appleyard and K. Paigen for their invaluable advice.

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† U.S. Public Health Service Training Fellow.

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[15] When a population of bacteria lysogenic for λv_1^+ is induced and at the same time superinfected by λv_1 with an average multiplicity of n, the distribution of fractions of bacteria superinfected with actual multiplicity i follows the Poisson equation, $P_{(i)} = n^i e^{-n}/i!$ When x is the initial number of prophage per bacterium, the ratio, R, of $\lambda v_1^+/\lambda v_1$ in the progeny population can be related as $R = \Sigma[xP_{(i)}/(x+i)]/1 - \Sigma[xP_{(i)}/(x+i)]$. A solution of this equation is not easily obtained, but one can make approximate computations within a biologically reasonable range of x, e.g., 1 to 10, against selected values of n, e.g., 2 to 10. The log-log plot of the resulting values of R against x for each n gives fairly linear relations which allow a relatively accurate interpolation for estimating x from the experimental values of R and n.

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*SEQUENTIAL REPLICATION OF BACILLUS SUBTILIS CHROMOSOME, I. COMPARISON OF MARKER FREQUENCIES IN EXPONENTIAL AND STATIONARY GROWTH PHASES**

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The mechanism of DNA replication is fairly well understood,[1-3] while that of chromosomal replication is rather poorly known. The main difficulty is our ignorance of the molecular structure of chromosomes. The results of Taylor, Woods, and Hughes[4] show a bipartite and semiconservative nature of plant chromosomes. So far as the sequence of chromosomal replication is concerned, autoradiographic analyses of chromosomes of higher organisms have revealed no over-all polarity of replication,[5, 6] except for a suggestive case in *Crepis*.[7] Existence of polarity in small regions, however, is quite likely.[5, 8] For the *E. coli* chromosome, Maaløe[9] proposed a model in which replication proceeds along the chromosome in an oriented fashion. Although there was some suggestive evidence favoring such a model,[9] no direct proof had been obtained.

Based on isotopic transfer experiments in the *Bacillus subtilis* transformation system, we have concluded that the replication of different markers in *B. subtilis* does not occur in a random-in-time fashion but has a regular oriented pattern.[10, 11] In recent reports by Nagata[12, 13] the pattern of duplication of λ prophage in synchronized cultures of an Hfr strain of *E. coli* K12 has been interpreted as evidence for a polarity of chromosome replication. A similar conclusion has been reached by Cairns from autoradiographic studies on DNA replication of *E. coli*.[14]

This paper presents experimental results which give further support to the polarity of replication of the bacterial chromosome. The experiments consist of measurements of relative frequencies of genetic markers in an exponentially growing cell population of *Bacillus subtilis*, using the genetic transformation system developed by Spizizen.[15] If chromosomal replication has a polarity, there should be a difference in the frequency of genes depending on their position on the chromosome. This is exactly what we found in the present experiments.

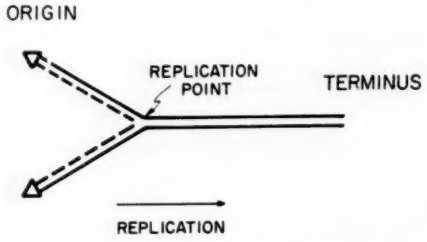

FIG. 1.—A replication model of the chromosome. Replication starts from one end (origin) of the chromosome, and proceeds toward the other end (terminus). Both strands are copied. The solid line represents the old strand and the broken one the newly synthesized strand. This model does not differ, in principle, from a circular chromosome model in which replication starts at a fixed point in one direction.

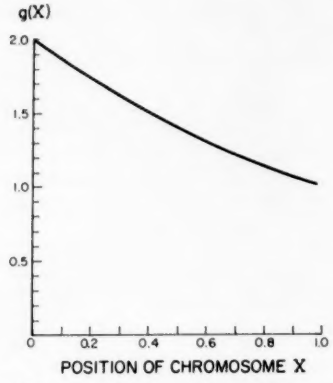

FIG. 2.—Relative frequencies of genetic markers at different positions on the chromosome in an exponentially growing population. The distribution (or mapping) function g(X) is not normalized; the integration of g(X) from X = 0 to X = 1 gives 1/ln2. This function was used for mapping markers (*see* Fig. 5).

The results gave clear evidence of an oriented replication of the chromosome and made it possible to construct a linear genetic map covering the whole range of the chromosome. Two small groups of linked genetic markers had been described in *B. subtilis* by joint transformation: the histidine-indole-tyrosine group[16-18] and the streptomycin-sporeless group.[19] The present experiments suggested the existence of another linked group, methionine and isoleucine, whose existence was then confirmed by joint transformation.

Materials and Methods.—Principles of the experiments: At the present stage of our knowledge of chromosomal structure[20,21] several alternative replication models are possible. The model which we chose to test experimentally is the one-end oriented, continuous replication model (Fig. 1). In this model, the chromosome replicates from one end (origin), proceeds continuously toward the other end (terminus) at an approximately uniform speed, and both strands replicate. For the sake of simplicity, the resting period in which no DNA synthesis occurs is assumed to be negligible.[9]

This particular model leads to several specific testable predictions. In an exponentially growing population without any synchrony of chromosomal replication, the frequency of chromosomes whose replication has just started is nearly twice as high as that of chromosomes which are about to complete their replication. Frequencies of chromosomes in the intermediate replication stages are intermediate between these two extremes (see appendix). This means that the frequency of a genetic marker in an exponentially growing population is a unique function of its location on the chromosome: i.e., the markers located toward the origin are more frequent than those toward the terminus. The frequency distribution of markers as a function of their position X on the chromosome is (see appendix): $g(X) = 2^{1-X}$.

The position X can vary from 0 (the origin) to 1 (the terminus); the corresponding values of $g(X)$ are 2 and 1, respectively. A graphic representation of the relation is given in Figure 2. If our model is correct, this relation can be used to map genetic markers in a linear order by experimentally measuring the relative frequency of different markers, $g(X)$, in an exponentially growing culture used as donor of DNA in a transformation experiment. Consequently, the function $g(X)$ is called the mapping function.

Experimental determination of marker frequency in a given transforming DNA preparation,

however, requires another condition. Since the efficiency of transformation varies from marker to marker due to differences in the integration efficiency,[22, 23] mere comparisons of numbers of transformants for different markers do not tell us the actual frequencies of the markers in the DNA preparation in question. This problem can be solved if we find a condition in which all markers are equally frequent. This condition will serve as the standard, and the relative frequency of markers in exponential cultures can be obtained by reference to the standard.

Conditions for equal marker frequency might be obtained by various methods. Removal of supplements from a growing culture of an amino acid-requiring mutant of *E. coli* might give the desired conditions.[9] In the present work, we chose the stationary phase as a possible example of the equal-gene-frequency situation. If all the cells in the stationary phase have complete, resting chromosomes, all markers should be equally frequent. Comparison of the relative transforming activity of DNA from exponential and stationary growth phases should disclose differences for different markers, if our replication model is right. If, on the other hand, the replication state or the age of the chromosome is distributed more or less similarly in the stationary phase as in the exponential phase, there should be no difference in marker frequency between DNA samples from the two growth phases.

A wild-type strain (W23) of *B. subtilis*[24] was used as the DNA donor strain throughout the work. Recipient mutants were isolated[11] from a Marburg strain (a derivative of strain 168 of Burkholder and Giles[25]) by an ultraviolet-penicillin method.[26, 27] Only mutants with low reversion frequencies, less than 10^{-7}, were used. DNA samples of the donor strain were prepared from cells in various growth stages. In order to stop further DNA replication, cells were heated at 60°C for 10 min immediately after sampling. They were chilled in ice, centrifuged, and suspended in a versene saline solution (0.15 *M* NaCl plus 0.1 *M* ethylenediamine tetracetate, pH 8.0) at a cell concentration of about 10^{10}/ml. The suspension was incubated at 37°C for 15 min with egg-white lysozyme (1 mg/ml). Lysis was completed by further incubation with duponol (final concentration 2 per cent) for 5 min at 37°C with constant shaking. DNA was prepared by a modification of Marmur's method[28] and was sterilized by keeping it in 70 per cent ethanol for several hours. The details of the methods will be described elsewhere.[11]

Transformation was performed mostly according to Anagnostopoulos and Spizizen.[24] In order to obtain competent recipient cells reproducibly, a growth curve was followed in each case by growing the recipient cells in a flask which allowed measurement of cell concentration without removal of samples. When the growth curve indicated the approach of the stationary phase, the cells were diluted tenfold with medium for a second growth phase[24] and shaken for 90 min at 37°C; then DNA was added. The cells were shaken further for 40 min and DNAase (5μg/ml) was added.

Experimental Results.—DNA was isolated from the donor strain at various times in the exponential and stationary growth phases (Fig. 3). Relative frequencies of transformants for various markers were compared for various samples of DNA from exponential and stationary phases. The results are presented in Tables 1 and 2. The results of Table 1 show that the ratios of transformants for one marker to those for another marker are independent of the DNA concentration used.

For each marker and for each DNA sample, the number of transformants obtained was normalized to the number obtained for the *met* (= methionine) marker. Then these normalized values were again normalized to the values for DNA sample #7, taken from a stationary phase culture (*see* Fig. 3). These final values show significant differences between different markers.

These differences indicate that at different stages in the growth of a culture there are significant differences between the frequencies with which different markers can be donated by transforming DNA. These differences should correspond, according to our hypothesis, to differences in the number of copies of the markers in the DNA and, hence, in the donor cells.

It is remarkable that for none of the markers tested (ten altogether, *see* Fig. 5) the normalized value for the exponential phase is more than twice the value in the

TABLE 1

TYPICAL DATA ON RELATIVE FREQUENCIES OF *leu*, *met*, AND *thr* MARKERS IN DNA FROM EXPONENTIAL AND STATIONARY GROWTH PHASES

DNA	Concentration	No. of Transformed Cells/10^7 Recipient Cells[a] *thr*	*leu*	*met*	Ratio *thr/met*		Ratio *leu/met*	
						1.62[d]		*1.22*
No. 3[b]	0.1 μg/ml	6,670	8,050	4,970	1.34	1.41[c]	1.62	1.59
No. 3	0.01	1,070	1,140	730	1.47		1.56	
No. 3	0.001	61	82	32	1.91[e]		2.58[e]	
No. 5	0.1	5,110	7,180	5,540	0.92	*1.17*	1.30	*1.07*
No. 5	0.01	1,350	1,720	1,270	1.06	1.02	1.35	1.39
No. 5	0.001	147	210	138	1.07		1.52	
No. 7	0.1	7,630	11,730	8,820	0.87	*1.00*	1.33	*1.00*
No. 7	0.01	1,670	2,580	2,070	0.81	0.87	1.25	1.30
No. 7	0.001	253	352	268	0.94		1.31	
No DNA		7	0	0				

[a] Each value is the average of two plate counts. The recipient strain used is a triple mutant, *leu-met-thr* (Mu8u5u5).
[b] The numbers of the DNA samples are the same as those shown in Figure 3.
[c] The average ratio.
[d] The average ratio relative to DNA No. 7 which is taken as 1.
[e] These figures were discarded because of the small number of transformed colonies per plate.

stationary phase. The marker with the highest frequency in the exponential phase is *ade*, the lowest are *met* and *ileu*. The ratio between these two classes of markers is 2, which is the maximal ratio predicted by the proposed model.

A more detailed analysis of early and late stationary phases was made using the two extreme markers, *ade* and *met* (Fig. 4). The results indicate that the higher frequency of the *ade* marker in the exponential phase decreases gradually and finally reaches the stationary value. This is the expectation from our postulate that the stationary chromosome represents the completed form.

The quantitative results fit the predictions so well that the proposed model of chromosome replication gains strong support. Alternative models will be considered in the discussion, but none of them are as satisfactory as the proposed model.

The genetic map of *B. subtilis* shown in Figure 5 was constructed by calculating the position (X) of each marker from the observed normalized value of that marker in the exponential phase using the mapping function. It should be noticed that those markers that had previously been shown to be linked[16–18] (*his*, *ind*, *tyr*) be-

TABLE 2

RELATIVE FREQUENCIES OF VARIOUS MARKERS TO *met* IN EXPONENTIAL AND STATIONARY GROWTH PHASES

Relative frequency of marker to *met*[a]	DNA Samples: Exponential No. 1	No. 2	No. 3	No. 4	Stationary No. 5	No. 6	No. 7[b]	Overnight DNA[c]
ileu/met			*1.04*		*0.97*		(*1.00*)	
leu/met	*1.37*	*1.43**	*1.24*	*1.12**	*1.05*	*1.03**	(*1.00*)	*1.05*
thr/met	*1.74*		*1.67*		*1.15*		(*1.00*)	
ade/met	*1.96*	*2.10**	*1.92*	*1.90**	*1.22*	*1.40**	(*1.00*)	*1.05*
his/met	*1.55*		*1.28*				(*1.00*)	*1.05*

[a] The recipient strains are triple mutants derived from a double mutant *leu-met* (Mu8u5): *leu-met-ileu* (Mu8u5u1); *leu-met-his* (Mu8u5u2); *leu-met-ade* (Mu8u5u6); *leu-met-thr* (Mu8u5u5).
[b] The ratios (marker/*met*) for DNA No. 7 are taken as 1 in this set of data and the other values are normalized to them. These values are the averages of several repeated measurements, except those marked with an asterisk, which are based on one observation only.
[c] Relative marker frequencies in DNA from an overnight culture are shown for additional comparison.

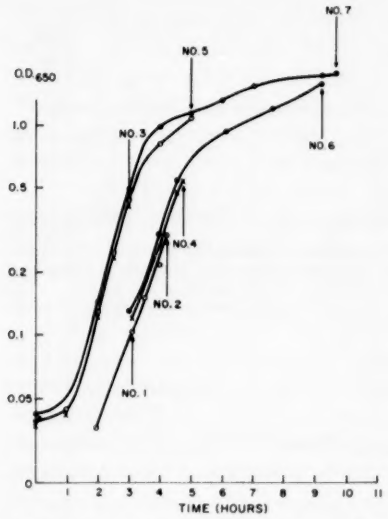

FIG. 3.—DNA samples from various growth stages of *B. subtilis* W23 in an enriched minimal medium. Cells were grown at 37°C with constant shaking in a minimal medium[24] supplemented with 0.05% casein hydrolyzate and 50 μg/ml of L-tryptophan. The volume was 1 liter for samples No. 1 to No. 4, 500 ml for No. 5 to No. 7. Cells of each sample were heated at 60°C for 10 min and DNA was prepared as described in the text.

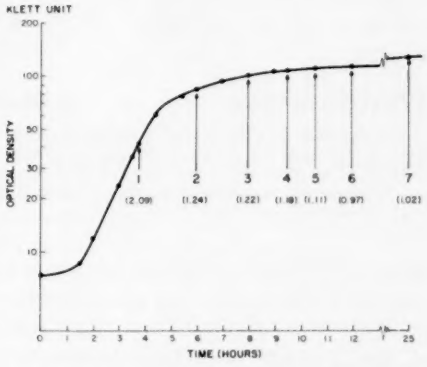

FIG. 4.—Detailed analysis of various stages of the stationary growth phase using *ade* and *met* markers. The figures in parentheses are the *ade/met* ratio. The culture and sampling conditions are similar to those of Figure 3. The recipient strain is a triple mutant strain, *leu-met-ade* (Mu8u5u6).

haved similarly in the present test, thus confirming the validity of the map (Table 3 and Fig. 5).

Extensive linkage tests were made among the markers used in the present experiment by searching for joint transformation of various combinations of the markers.[29] Besides the previously known *his-ind-tyr* linkage group, a new linkage between *met* and *ileu* was discovered (Table 4). This is in complete agreement with the proposed map (Fig. 5).

Discussion.—The method and results reported here and in a previous note[10] support an oriented replication of the *B. subtilis* chromosome and present a new opportunity to study the genetic structure and replication mechanism of chromo-

TABLE 3

MARKER FREQUENCIES OF *tyr*, *ind*, AND *his* IN VARIOUS STAGES OF GROWTH

DNA	No. of Transformed Cells[c]			Ratio			
	tyr	*ind*	*his*	*tyr/his*		*ind/his*	
No. 1[a]	243	281	291	0.84	*0.85*	0.97	*0.94*
No. 2	262	279	257	1.02	*1.03*	1.08	*1.05*
No. 3	437	484	427	1.02	*1.03*	1.13	*1.10*
No. 4	337	378	351	0.96	*0.97*	1.07	*1.04*
No. 5	752	807	760	0.99	*1.00*	1.06	*1.03*
No. 6	606	598	599	1.01	*1.02*	1.00	*0.97*
No. 7	941	999	955	0.99	*(1.00)*[b]	1.03	*(1.00)*[b]

[a] The numbers of the DNA samples are the same as those shown in Figure 3.
[b] The *tyr/his* and *ind/his* ratios of No. 7 DNA were taken as 1.
[c] The recipient strain used was a triple mutant, *tyr-ind-his* (My1y1u7).

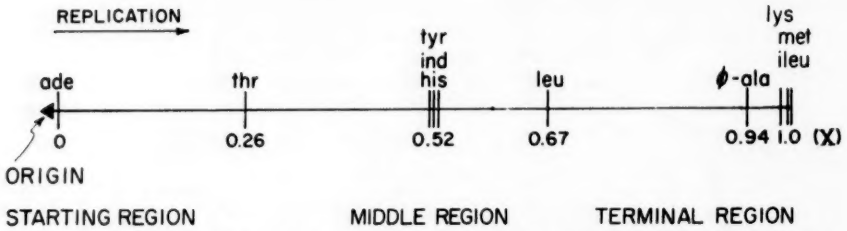

FIG. 5.—A genetic map of *B. subtilis* obtained by the method described in the text. The position of each marker on the chromosome is obtained from the average of several experimental values. The gene orders for *tyr*, *ind*, and *his*, and for *ileu* and *met*, are not yet known. The markers *phe* and *lys* were mapped by using the following double mutants as recipient strains: *leu-phe* (Mu8u7), and *lys-phe* (Mu12u17).

somes. Excluded are the models assuming random-in-time replication of different regions of the chromosome and those in which only one chain of DNA is copied, while the other chain remains single over a considerable length. The latter class of models is also made unlikely by isotopic transfer experiments[2] and autoradiographic studies[14] in *E. coli*.

Two modified forms of the oriented replication model, however, cannot be excluded completely. One is the "subunit-model" in which the chromosome consists of subunits which replicate more or less independently of one another. The replication of each subunit should, however, have polarity to satisfy the present results. In this scheme, the *ade* locus would be located close to the origin of one of the subunits, and *met* and *ileu* close to the terminus of some subunit. A subunit structure of chromosomes, in which the replication of each subunit proceeds in sequential order from one end of the chromosome to the other, is not distinguishable from the model proposed in this paper and does not require special consideration at the moment. In another model, replication would start from both ends of the chromosome or of its subunits (two-end model). In this case, the markers that we have mapped in the region close to the origin would physically occupy either end of the chromosome, and those mapped in the terminal region would occupy the middle region.

These two schemes would drastically change the genetic map. Although a subunit model cannot be excluded, the behavior of linked markers in the present experiments indicates that the subunits, if any, may be fairly large in size. The two-end model cannot readily be excluded by experiments of the present type unless more markers are accumulated and mapped. However, two lines of evidence on the

TABLE 4

A NEW LINKAGE GROUP (METHIONINE-ISOLEUCINE) IN *B. subtilis*

Conc. of DNA (μg/ml)	No. of Transformed Cells per 10^6 Recipient Cells[d]					Per Cent Cotransfer Index[a]	
	leu	*ileu*	*met*	*ileu-met*	*ileu-leu*	*ileu-met*	*ileu-leu*
0.04	334[b] ± 13[c]	158 ± 7	162 ± 2	27 ± 2	0	9.6	<0.2
0.008	229 ± 25	106 ± 3	100 ± 19	18 ± 5	0	9.6	<0.3
0.004	136 ± 6	48 ± 5	52 ± 11	9 ± 1	0	9.0	<0.5
No	0	0	0	0	0	...	...

[a] The cotransfer index was calculated according to Nester and Lederberg[16] and is expressed in percentage.
[b] Average number of colonies from 3 plates.
[c] Extreme values are shown to indicate the extent of errors.
[d] The recipient strain used was a triple mutant *leu-met-ileu* (Mu8u5u1).

E. coli chromosome tend to exclude these possibilities and support the one-end continuous replication model. One is an autoradiographic study by Cairns, who observed only one replication "fork" per chromosome.[14] The other is a study by Nagata,[12, 13] showing that in synchronously growing cultures of Hfr strains of *E. coli* K12 there is a correlation between the position of the prophage λ on the host chromosome and the time of prophage replication.

The equal frequency of markers at the stationary phase predicts that the chromosome will replicate synchronously when cells in the stationary phase are transferred to a fresh medium. In the previously reported cases of cell synchrony in bacteria,[30,31] it was an unsettled question whether or not chromosome synchrony accompanied cell synchrony. It is now possible to check the degree of chromosome synchrony in these cases by comparing the relative frequency of the two end markers or of any two markers located at different positions on the chromosome.

The age (replication-stage) distribution of chromosomes in the exponential phase described here assumes that no sizable fraction of the generation time is spent in the resting stage in which the chromosome does not engage in replication. In other words, the mapping function holds for growth conditions in which the resting period is negligible. This appears to be true for the present experiments, because the ratio of *ade* to *met* gives the value of 2. In general, however, the frequency ratio of any two markers in the exponential phase will be smaller the larger the fraction of the generation time occupied by the resting period, since a part of the population will have different gene frequencies, as described in this paper, while the resting part of the population will have equal frequencies for all markers. Using the two extreme markers, *ade* and *met*, it should be possible to detect the presence and to calculate the length of the resting phase in other culture conditions.[32]

As previously discussed by others,[9, 31, 33] there should be some regulating mechanism that controls chromosomal replication so that the normal DNA:mass ratio can be kept fairly constant in various growth conditions. It is attractive to assume that there is at the tip of the chromosome a region which signals the start of the chromosomal replication. The activation for such a signal gives an analogy of a repressor-inducer type mechanism.

Summary.—Relative frequencies of various genetic markers in the DNA of *B. subtilis*, strain W23, in exponential and stationary growth phases were compared using a transformation system. If the chromosome replication has a polarity, the frequency of each marker in the exponential phase should be a function of its location on the chromosome. The results indicate that such polarity exists in *B. subtilis*. On the basis of the results, a genetic map has been constructed in which the adenine marker is located near the point of origin, from which the chromosome starts replicating, and methionine and isoleucine near the terminus. The results also indicate that chromosomes in the stationary phase are in completed form.

Appendix.—The replication model described in this paper (Fig. 1) leads to the following quantitative description of the population.

In an exponentially growing population, the frequency of chromosomes which have replicated up to a position x is described by

$$f(x) = (\ln 2)2^{1-x} \qquad (1)$$

The distribution function is equivalent to the age distribution function of cells solved by Powell[34] for organisms which replicate regularly by binary fission.

The frequency of a particular marker which occupies a position X of the chromosome is given by

$$g(X) = \int_0^X f(x)dx + 2\int_X^1 f(x)dx = 2^{1-x} \quad (2)$$

Here the function $g(X)$ is not normalized, so that the total number of markers is equivalent to $1/\ln 2$, while the total number of chromosomes is taken as 1 (equation 1). For graphic representation, see Figure 2. A detailed presentation of the above functions and their applications to other cases will be presented elsewhere.[32]

We are grateful to Drs. M. Meselson and F. W. Stahl, and Mr. C. Neuman for stimulating discussions. This investigation was supported by research grants from the National Science Foundation (GB-39 and GB-577).

* The abbreviations, *ade*, *his*, *ileu*, *ind*, *leu*, *lys*, *met*, *ϕ-ala* (or *phe*), *thr*, and *tyr* were used for mutants requiring adenine, histidine, isoleucine, indole, leucine, lysine, methionine, phenylalanine, threonine, and tyrosine, respectively.

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ROLE OF CHLOROPLAST FERREDOXIN IN THE ENERGY CONVERSION PROCESS OF PHOTOSYNTHESIS

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Chloroplast ferredoxin,[1-4] a water soluble iron protein with a redox potential[5] ($E_0' = -432$ mV, pH 7.55) close to that of the hydrogen electrode, is the most electronegative constituent isolated so far from the photosynthetic apparatus of green plants. Reduced ferredoxin has recently been identified[6] as the earliest, chemically defined reductant formed by chloroplasts at the expense of absorbed radiant energy. These properties are compatible with a role for ferredoxin as an electron carrier in cyclic (eq. 1) and noncyclic (eq. 2) photophosphorylation[7-10]—the two components of the photosynthetic energy conversion process that produces the assimilatory power ($TPNH_2$ and ATP[11]) to drive carbon assimilation by chloroplasts.[12-14]

$$ADP + P_i \xrightarrow[\text{chloroplasts}]{h\nu} ATP \qquad (1)$$

$$2TPN + 2ADP + 2P_i + 2H_2O \xrightarrow[\text{chloroplasts}]{h\nu} 2TPNH_2 + 2ATP + O_2 \qquad (2)$$

This article reports evidence for a role of ferredoxin in an endogenous type of cyclic photophosphorylation by chloroplasts (eq. 1), which proceeds anaerobically without added cofactors. A requirement for ferredoxin (then called "TPN-reducing factor"[1]) in noncyclic photophosphorylation (eq. 2) was already observed when this process was first discovered,[8] and this requirement has since been further documented.[9, 15-17] Evidence was also obtained several years ago[3] for a requirement of a "TPN-reducing factor" (i.e., ferredoxin) in what is now called pseudocyclic photophosphorylation[18] by chloroplasts, but the significance of these earlier observations for the mechanism of energy conversion in photosynthesis has only now been realized.

Methods.—"Broken" chloroplasts[28] (P_{1s} or C_{1s}) were used in all experiments. The incandescent light used for illumination was filtered through a Jena G1 or G2 filter, which cut off light of wavelength shorter than 500 mμ. The ATP formed was estimated by the method of Hagihara and Lardy.[19] Other experimental procedures were the same as those used previously.[28]

Results and Discussion.—In the mechanisms currently envisaged in our laboratory,[10, 20] cyclic and noncyclic photophosphorylation share the same primary photochemical act which gives rise, within the chloroplasts, to an electron flow from an excited chlorophyll molecule to an endogenous, primary electron acceptor. If, as recent results have shown,[6] ferredoxin is the earliest primary electron acceptor that can now be isolated following the photochemical act, then it follows that photoreduction of ferredoxin should be a common feature of the electron flow pathways of either cyclic or noncyclic photophosphorylation. The two pathways would differ in the electron acceptors beyond ferredoxin. In the case of noncyclic photophosphorylation, the electrons from ferredoxin would be transferred to TPN,

whereas in the case of cyclic photophosphorylation they would "cycle" back to "electron-deficient" chlorophyll molecules[10, 20] *via* a chain of endogenous electron carriers. (The transfer of electrons from reduced ferredoxin to TPN occurs only in the presence of a flavoprotein reductase which is contained in the chloroplasts.[21])

If this formulation of the role of ferredoxin is correct, then a transition from noncyclic to cyclic photophosphorylation should be regulated by the availability of TPN as the electron acceptor. Figure 1 shows that this was experimentally observed. The lowermost curve represents cyclic photophosphorylation in the absence of TPN. The rate of ATP formation by cyclic photophosphorylation was lower than that by noncyclic photophosphorylation in the presence of TPN. Noncyclic photophosphorylation continued at the higher rate as long as a supply of oxidized TPN was available (uppermost curve). But when all of the available TPN was reduced (middle curve), the more rapid noncyclic photophosphorylation came to an end and the slower cyclic photophosphorylation took its place. The rate of this "delayed" cyclic photophosphorylation was the same as the rate of the initial cyclic photophosphorylation in the absence of TPN (compare the slopes of the middle and lowermost curves in Fig. 1). Where TPN was added (uppermost and middle curves of Fig. 1), its photoreduction was measured spectrophotometrically by absorption at 340 mμ. The quantities of $TPNH_2$ found confirmed the stoichiometry of noncyclic photophosphorylation[8] ($TPNH_2/ATP = 1$).

The experiments represented by Figure 1 were carried out under aerobic conditions. Forti and Jagendorf,[22] and Black *et al.*[23] have observed that under these conditions ferredoxin ("PPNR") stimulates an endogenous photophosphorylation which proceeds in the absence of added cofactors, but is dependent on, and consumes, molecular oxygen as the terminal electron acceptor. Forti and Jagendorf[22] interpreted their data as ruling out the existence in chloroplasts of a truly endogenous cyclic photophosphorylation, i.e., a phosphorylation that would proceed without the participation of molecular oxygen as the terminal electron acceptor. Evidence was needed, therefore, to show that ferredoxin could catalyze an endogenous cyclic photophosphorylation under anaerobic conditions, when oxygen was initially excluded and its photochemical production effectively prevented.

Evidence for an endogenous ferredoxin-catalyzed cyclic photophosphorylation, which proceeds anaerobically without the addition of other cofactors, was obtained after first establishing the experimental conditions under which this type of photophosphorylation can be observed. These experimental conditions include a relatively low light intensity (illumination higher than 10,000 lux was inhibitory), an optimum pH about 8.2, and the use of *p*-chlorophenyldimethyl urea (CMU) as an inhibitor of oxygen evolution. The significance of these experimental conditions will be discussed in a subsequent publication.

Table 1 shows the dependence of anaerobic cyclic photophosphorylation on the addition of ferredoxin. In other experiments, it was found that under anaerobic conditions the ferredoxin requirement was much greater (up to tenfold) for cyclic photophosphorylation than for the noncyclic type.

As shown in Table 2 the anaerobic cyclic photophosphorylation catalyzed by ferredoxin was strongly dependent on the presence of CMU. In the absence of CMU there was no significant photophosphorylation. The addition of 10 μg of CMU in-

creased the rate of photophosphorylation about sevenfold, but 100 μg of CMU was inhibitory.

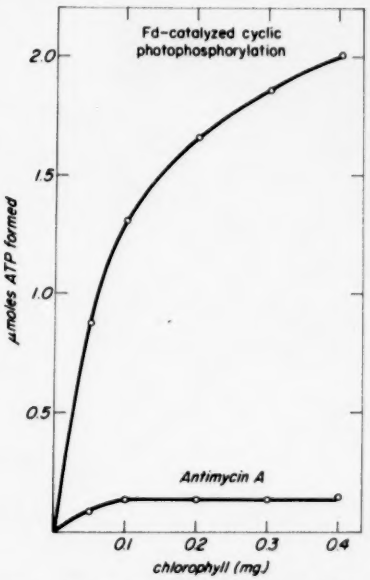

FIG. 1.—Transition from noncyclic to cyclic photophosphorylation, catalyzed by ferredoxin (Fd), as influenced by TPN availability.

The reaction mixture contained in a final volume of 4.5 ml, broken chloroplasts (C_{1b}) containing 300 μg of chlorophyll, 1,500 μg of spinach ferredoxin and the following in μmoles: tris buffer pH 8.3, 150; $MgCl_2$, 7.5; ADP, 15; $K_2HP^{32}O_4$, 15; and TPN as indicated. The reaction was carried out in 1 cm optical path cuvettes under air at 15°C. Illumination was by incandescent light (10,000 lux) filtered through a Jena G2 filter. At the indicated times, 0.5 ml aliquots of the reaction mixture were withdrawn for ATP determination and 0.3 ml for $TPNH_2$ determination (see text). The ATP produced is plotted as μmoles ATP in 1.5 ml of reaction mixture.

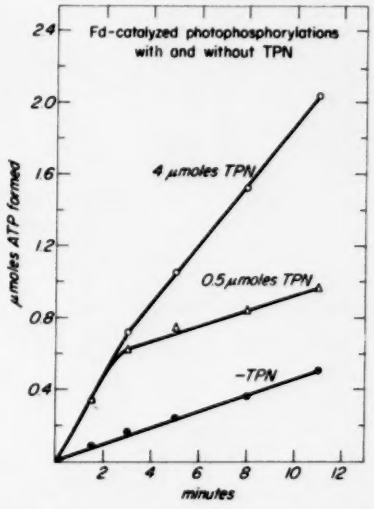

FIG. 2.—Experimental conditions were as described in the legend for Table 1 except that 1,500 μg of spinach ferredoxin were used throughout and a variable amount of chlorophyll as indicated. 10 μg of antimycin A were added where indicated.

A notable feature of ferredoxin-catalyzed cyclic photophosphorylation was its sensitivity to antimycin A over a wide range of chlorophyll concentration (Fig. 2). Sensitivity to antimycin A has been observed in the endogenous anaerobic cyclic photophosphorylation by chromatophores of the purple bacteria, *Rhodospirillum rubrum*[24, 25] and *Chromatium*.[26] The inhibition of cyclic photophosphorylation in chromatophores does not occur in the presence of catalytic amounts of phenazine methosulfate,[24, 27] which evidently serves as a bypass around the site of antimycin A inhibition. Interestingly enough, the usual type of cyclic photophosphorylation in chloroplasts—measured in the presence of added cofactors and at relatively high chlorophyll concentrations—was also found to be resistant

TABLE 1

EFFECT OF FERREDOXIN ON CYCLIC PHOTOPHOSPHORYLATION IN AN ATMOSPHERE OF ARGON

Ferredoxin added (μg)	ATP formed	
	cpm	μmoles
0	23	0.00
30	146	0.02
75	410	0.07
150	789	0.14
300	2,109	0.37
750	7,404	1.29
1,500	10,816	1.88

The reaction mixture contained, in a final volume of 1.5 ml: spinach ferredoxin as indicated, *p*-chlorophenyldimethyl urea (CMU), 2 μg; washed broken chloroplasts containing 100 μg of chlorophyll and the following in μmoles: tris buffer pH 8.0, 50; $MgCl_2$, 2.5; ADP, 5; $K_2HP^{32}O_4$, 5. The reaction was carried out in Warburg vessels in an atmosphere of argon at 15°C. Illumination was by incandescent light (10,000 lux) filtered through a Jena G2 filter.

TABLE 2

EFFECT OF *P*-CHLOROPHENYLDIMETHYL UREA (CMU) ON FERREDOXIN-CATALYZED CYCLIC PHOTOPHOSPHORYLATION IN AN ATMOSPHERE OF ARGON

CMU added (μg)	ATP formed (μmoles)
None	0.21
0.1	0.21
1.0	0.53
10	1.57
100	0.02

Experimental conditions were as described in the legend for Table 1 except that 1,500 μg of spinach ferredoxin were used throughout. CMU was added as indicated.

to antimycin A inhibition.[28] Since, in mitochondria,[29, 30] antimycin A inhibition is considered to be indicative of the participation of cytochrome *b* in electron transport, the sensitivity to antimycin A suggests a possible participation of the cytochrome b_6 component of chloroplasts[31, 32] in the ferredoxin-catalyzed cyclic photophosphorylation.

Concluding Remarks.—A previously puzzling feature of cyclic photophosphorylation in isolated chloroplasts, a feature which distinguished it from cyclic photophosphorylation in bacterial chromatophores, was a dependence on an added electron carrier such as vitamin K or phenazine methosulfate. A possible, though heretofore experimentally unsupported, explanation of this difference was that chloroplasts, but not chromatophores, automatically lost a soluble constituent in the process of isolation. The present findings point to chloroplast ferredoxin as being the water-soluble constituent of cyclic photophosphorylation which is, at least in part, lost from chloroplasts when they are removed from the cell. However, it is still premature to say what role bacterial ferredoxins[5] play in the mechanism of bacterial photophosphorylation. Differences between the two systems require further study. Thus, it has been found that the pyridine nucleotide-reducing system in chromatophores differs from that in chloroplasts. Chromatophores photoreduce DPN in preference to TPN and do so without the addition of a soluble cofactor such as ferredoxin.[26]

In chloroplasts, ferredoxin appears to be a branching point in the electron transport systems that result either in cyclic or in noncyclic photophosphorylation. Since TPN is required for noncyclic photophosphorylation but not for cyclic photophosphorylation,[33] it follows that when the photoreduced ferredoxin is reoxidized by TPN (with the aid of an appropriate flavoprotein enzyme[21]), noncyclic photophosphorylation would result. When oxidized TPN is unavailable

as an electron acceptor, the photoreduced ferredoxin would be reoxidized by a bound component of the grana, and cyclic photophosphorylation would result. It is thus possible to envisage that the availability of TPN as an electron acceptor would serve as a physiological regulator between cyclic and noncyclic photophosphorylation. TPN availability would, in turn, be regulated by the reoxidation of $TPNH_2$ in the course of reductive carbon assimilation (cf. ref. 8).

It is interesting to note that, although reduced ferredoxin is nonenzymatically oxidized by oxygen,[5] an appreciable leakage of electrons to O_2 (is always present around the chloroplasts *in vivo*) is prevented by the strong affinity of reduced ferredoxin, (*a*) for the TPN-reducing system, and (*b*) for the grana-bound electron carriers of cyclic photophosphorylation. We have observed, even under aerobic conditions, a cyclic photophosphorylation in isolated chloroplasts that was essentially independent of molecular oxygen as the terminal electron acceptor. A more detailed discussion of this point will be presented elsewhere.

* Aided by grants from the U.S. Public Health Service and the Office of Naval Research.

[1] Ferredoxin of chloroplasts is the same substance as the methemoglobin-reducing factor of Davenport, Hill, and Whatley,[2] the TPN-reducing factor of Arnon, Whatley, and Allen,[3] and the photosynthetic pyridine nucleotide reductase (PPNR) of San Pietro and Lang.[4] This change in nomenclature, discussed in more detail elsewhere,[5] is based on the recently discovered chemical and functional similarities of ferredoxins from bacteria and chloroplasts.

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[33] Our recent experiments, with improved experimental methods, do not support our earlier conclusion[3] that TPN is also required for cyclic photophosphorylation.

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*THE DEFECTIVENESS OF ROUS SARCOMA VIRUS**

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The Bryan high-titer strain of Rous sarcoma virus (RSV) produces foci of transformed cells after infecting monolayers of chick embryo cells in tissue culture. The transformed cells take on the typical rounded appearance of cultivated Rous sarcoma cells and are no longer restricted to growth in a monolayer. When a stock of RSV is diluted beyond the end point for focus formation, another virus can readily be isolated which has been called Rous-associated virus (RAV).[1] RAV does not cause a noticeable morphological change in cells, but it induces resistance to RSV within a few days, and this resistance is the basis for the assay of RAV. RAV is indistinguishable from RSV in heat sensitivity, cellular site of maturation, growth rate, and immunological specificity.[1] It is known to differ from RSV only in its failure to produce either foci in tissue culture or sarcomas in the chicken. It does, however, produce leukosis in chickens, and can be considered a virus of the avian leukosis complex. In view of its close relationship to RSV and its presence in RSV stocks in higher titer than RSV itself, it seemed unlikely that RAV was a mere accidental contaminant of the RSV stock. A study was undertaken to clarify the relationship of the two viruses. Attempts were made to isolate a stock of RSV free of RAV by picking single foci of transformed cells. Although the transformed cells present in such foci multiplied indefinitely and maintained their distinctive morphology, they failed to produce either RSV or RAV. However, when RAV was added to such cells, they quickly produced large amounts of both RAV and RSV. We conclude from these observations that RSV is a defective virus which can only produce mature virus in the presence of a helper virus such as RAV.

Material and Methods.—RSV stock and assay: The high titer strain of RSV was used in the present studies. The stock was obtained from the medium of heavily infected monolayers of chick embryo cells. The medium was subjected to sonic vibration at 9 kc to disperse virus aggregates. The virus titer was assayed by focus formation on chick embryo cells.[2] The RSV stock contained 5×10^6 focus forming units (FFU) of RSV per ml and about 5×10^7 infectious units of RAV per ml (see below).

RAV stock and assay: RAV was isolated from a stock of RSV and was purified twice by limiting dilution *in vitro*. The stock used was obtained by disrupting infected chick embryo cells by sonic vibration. The virus was assayed by its interference with RSV infection as described

in a previous paper.[1] The titer was calculated from the terminal dilution which induced interference, and was expressed in terms of infectious units. The RAV stock contained about 10^8 infectious units of RAV per ml. No evidence for the presence of RSV could be found upon inoculation of the stock into chickens, chick embryos, and cultivated chick embryo cells.

Other viruses: Avian lymphomatosis virus (RIF)[2] was obtained from the plasmas of congenitally infected chickens. Avian myeloblastosis virus was obtained after one passage in tissue culture of virus from plasmas of infected chickens. The Bryan standard strain of RSV (H_2C_1 strain) which had been purified by isolation of a single focus was obtained from Dr. John Bader.

Antiserum to RAV: Four-week-old chickens were infected intravenously with 10^6 infectious units of RAV and were bled for antibody one month later. At a dilution of 1:1,000 these sera reduced the titer of both RSV and RAV by a factor of 1,000 in 40 min at 37°C.

Chick embryo cell culture: The techniques for preparing cultures of chick embryo cells have been described in detail elsewhere.[2] The only modification was in the medium used to grow cells which had been transformed by infection with RSV. These cells could be maintained more readily if overlaid with a medium containing 0.4% agar. All media contained 199 as the basic ingredient plus 10% tryptose phosphate broth and 5% calf serum.

Results.—The absence of virus production in foci of transformed cells: As the first step in studying the relationship between RSV and RAV, an attempt was made to obtain stock of RSV which was free of RAV. The RSV stock containing RAV was added to chick embryo cultures at a very high dilution so that only 2 or 3 foci appeared per plate in order to minimize likelihood of contamination of the foci by RAV. The cultures were overlaid with an agar medium containing anti-RAV serum as a further precaution against contamination of the RSV foci by RAV. After allowing 7 days for the development of foci, trypsin was added to loosen the attachment of the cells to the dish; the isolated foci were removed with a capillary pipette and added to 10^6 normal chick embryo cells. Serial transfers of the cultures were made at intervals of 3–4 days. At each of the early transfers an aliquot of the cells was diluted and plated on normal chick embryo cells to determine the number capable of growing into foci. Before each transfer the supernatant fluid was harvested and assayed for RSV and RAV.

In the course of many such experiments, it was found that more than 80 per cent

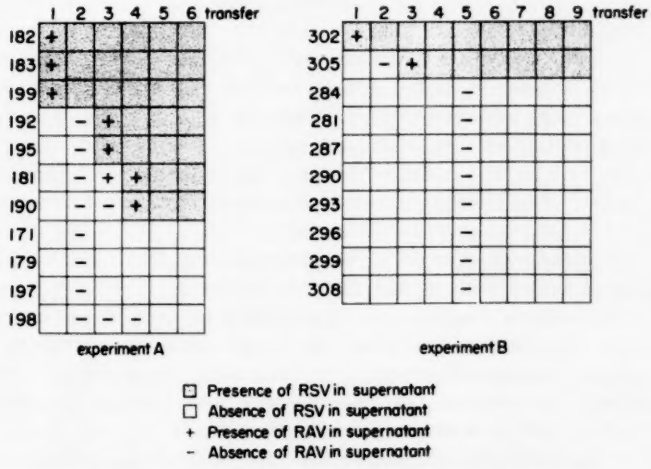
1 2 3 4 5 6 transfer
182 + + + + + +
183 + + + + + +
199 + + + + + +
192 − + + + + +
195 − + + + + +
181 − + + + + +
190 − − + + + +
171 − + + + + +
179 − + + + + +
197 − + + + + +
198 − − − + + +
experiment A
1 2 3 4 5 6 7 8 9 transfer
302 + + + + + + + + +
305 − + + + + + + + +
284 − + + + + + + + +
281 − + + + + + + + +
287 − + + + + + + + +
290 − + + + + + + + +
293 − + + + + + + + +
296 − + + + + + + + +
299 − + + + + + + + +
308 − + + + + + + + +
experiment B
Presence of RSV in supernatant
Absence of RSV in supernatant
+ Presence of RAV in supernatant
− Absence of RAV in supernatant

FIG. 1.—Pattern of RSV production from transferred foci.

TABLE 1

NUMBER OF TRANSFORMED CELLS CAPABLE OF GROWING INTO FOCI

Focus no.	Foci produced after plating original focus	Foci produced by plating at transfer (per 10^6 cells)			
		1st transfer	2nd transfer	3rd transfer	4th transfer
Virus producers					
302	52	1,100 (v)*	39,000 (v)	...	...
305	80	1,200	7,200	31,400 (v)	>190,000 (v)
Nonproducers					
281	160	2,300	55,000	45,000	>100,000
284	18	24	4,700	25,000	>100,000
287	80	380	12,000	54,000	25,000
290	90	460	12,000	...	40,000
293	12	28	140	555	1,100
296	10	72	1,900	4,700	40,000
299	4	70	224	1,500	5,100
308	50	78	810	1,600	5,800

* (v) = RSV demonstrated in the medium.

Individual foci were picked and added to cultures of uninfected chick embryo cells. The mixed cultures were transferred serially. At each transfer, an aliquot of cells was plated separately to determine the number able to initiate a focus. The cultures presented in this table are the same as those of experiment *B*, Fig. 1.

of the original foci failed to release either RSV or RAV at the first transfer despite the fact that some of the cultures contained over 1,000 transformed cells. Cells with the characteristic morphology of transformed Rous sarcoma cells but not producing virus will be referred to as nonproducing (NP) cells. The presence or absence of virus in two experiments is shown in Figure 1, and the number of transformed cells present during the first four transfers of one of these experiments is shown in Table 1. The transformed NP cells clearly had a growth advantage over the normal cells, since they formed an increasingly large fraction of the population with continuing transfer (Table 1). It can be seen that the transformed cells derived from many individual foci failed to produce virus at any time over as many as 9 transfers (Fig. 1), even though they already represented over 10 per cent of the population in some cultures by the 4th transfer and constituted a majority in some of the cultures of the 9th transfer. Cultures which failed to release virus into the medium also failed to yield virus when the cells were disrupted by sonic vibration. The medium of the NP cells had no inhibitory effect on the infectivity of RSV.

A further attempt was made to detect virus production from NP cells which had been transferred 3 times, by exposing them to 5,000 r of X irradiation and plating them on normal chick embryo cultures. The dose of X rays was large enough to block cell division, but not large enough to affect virus release.[3, 4] The plating of irradiated cells is a highly sensitive technique for detecting virus production, because the released virus has an immediate opportunity to contact susceptible cells. The results of this experiment are presented in Table 2. It can be seen that the cells of all NP cultures which failed to release detectable virus into the medium during the course of the experiment, also failed to register as infective centers after irradiation. The cells of virus-producing culture #302, which was included as a control for the sensitivity of the technique for detecting virus-releasing cells, induced focus formation as effectively after X irradiation as before. The results show, therefore, that even the most sensitive technique available for the detection of virus fails to reveal any virus production by the NP cells.

Some of the foci which did not produce RSV in the early transfers began to release RSV spontaneously after several transfers. Every one of the total of 53 such foci

TABLE 2

INABILITY OF NP CELLS TO INITIATE FOCI AFTER X IRRADIATION

Culture no.	Foci produced per 3.6×10^5 cells		RSV in medium before transfer (FFU/0.1 ml)
	Unirradiated	Irradiated	
281	16,600	0	0
284	9,200	0	0
287	19,400	0	0
293	200	0	0
296	1,900	0	0
299	550	0	0
305	11,200	0	0
308	560	0	0
302*	18,800	17,000	3,800

* A virus releasing control culture.
At the third transfer the media from mixed cultures of NP and normal cells were assayed for RSV and the cells were plated for focus formation before and after receiving 5000 r of X irradiation.

found to date began to release RAV at the same time or slightly before releasing RSV, and the titer of RAV was always somewhat higher than that of RSV. There was not a single case of RSV production in the absence of RAV production.

RSV production after infection of NP cells by RAV: The consistent failure to obtain RSV production without RAV production suggested that RAV might be essential to the production of mature RSV. To investigate this possibility further, RAV was added to NP cultures and the type of virus released into the medium determined. It was found that the addition of RAV to 42 NP cultures was followed by the release of infectious RSV and RAV in every case. (The simple mixing of RAV with the cell-free medium from such cultures did not unmask a putative RSV, showing that RAV actually initiated the production of RSV by the cells.) The daily yield of RSV following the addition of RAV was determined in three of the NP cultures, and the results are shown in Figure 2. RSV release became detectable in large amounts within a day after the addition of RAV, and continued with only a slight increase in rate thereafter.

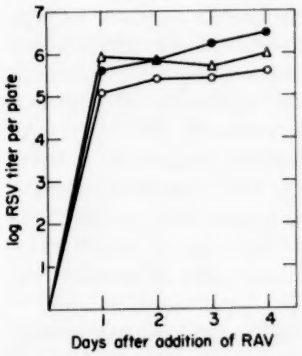

FIG. 2.—Release of RSV following the addition of RAV to NP cultures. 5×10^7 infectious units of RAV in 0.5 ml were added to monolayers of NP cultures. There were 4.9×10^4 NP cells in #287 (●) 1.8×10^4 NP cells in #290 (○), and 3.1×10^4 NP cells in #299 (△). Titer of RSV in the supernatant medium was assayed at daily intervals.

The unfailing initiation of infectious RSV production by RAV superinfection can be explained if we assume that RSV is incapable of producing infective progeny in solitary infection, and that RAV plays the role of a helper virus. This assumption is supported by the finding that the spontaneous onset of RSV production in some foci after several transfers was always preceded or accompanied by RAV production. The presence of RAV in such cases can plausibly be attributed to contamination of the original foci by the progeny of RAV present in the initial inoculum despite the precautions taken to prevent such an occurrence.

If RAV production by transformed cells of the foci were the result of accidental contamination, it would be expected that normal cells from some of the interfocal areas of the original plates would also have been contaminated. The proportion

of such interfocal areas which produces RAV should be about the same in a given experiment as the proportion of transformed foci which produces RAV and RSV, but the former should produce only RAV. As part of experiment *A* of Figure 1, 8 interfocal areas were picked, transferred, and tested for virus production. Cells from 5 of the 8 interfocal areas produced RAV, and only RAV, at the 3rd transfer. Since 7 out of the 11 foci in this experiment eventually produced both RAV and RSV, the findings support the suggestion that spontaneous RSV production was due to contamination with RAV. When rigorous precautions were taken to avoid contamination (exp. *B*, Fig. 1), most NP foci could be maintained as nonproducers indefinitely.

The effect of a variety of agents other than RAV on initiating RSV production by NP cells was tested. Successful initiation has been attained with all the viruses of the avian leukosis complex thus far tested, including a strain of visceral lymphomatosis virus (RIF), myeloblastosis virus, and an RAV-like agent isolated from Bryan's standard strain by RSV.

Absence of RSV production in the early stages of solitary infection: The foregoing experiments have shown that RSV is not produced by the cells on fully developed foci following solitary infection with RSV. Since these foci had been picked one week after infection, and had developed in the presence of neutralizing antibody, it could not be ascertained whether transient RSV production had occurred at an early stage of infection. The following experiment was therefore carried out to detect such early virus production by singly infected cells.

Approximately 100 FFU of RSV were adsorbed for one hr to chick embryo cell monolayers containing 10^6 cells. These will be referred to as RSV-infected cultures, since the low multiplicity of infection assured solitary RSV infections at the cellular level. Half of the cultures were then infected with 10^7 infectious units of RAV so that each RSV-infected cell was superinfected with RAV. All cultures were washed and overlaid with agar medium containing antibody to RAV. On each subsequent day the agar was removed from one RSV-infected culture and one RAV-superinfected culture and the cells were suspended with trypsin. An aliquot of the suspended cells from each culture was X-irradiated with 5,000 r. Irradiated and unirradiated cells were centrifuged, resuspended in medium and plated for focus formation on chick embryo cells.

It was anticipated that unirradiated RSV-infected cells would register as focus formers regardless of whether they produced virus since they could multiply as transformed cells. The irradiated RSV-infected cells, however, could only initiate foci if they released virus. Their failure to initiate foci could be interpreted as a failure to release virus only if X irradiation did not suppress focus formation by the cells superinfected with an excess of RAV, which were expected to release RSV. It can be seen in Table 3 that the unirradiated cells from cultures infected with 100 FFU of RSV produced about 100 foci when plated one day after infection. The number of foci produced by the RSV-infected cells increased by a factor of about 60 in the next 4 days, indicating an average generation time for the NP cells over this period of about 18 hr. Irradiation reduced the number of foci produced by these cells more than 100-fold during the first 3 days, and more than 30-fold on the 4th and 5th days.

The RSV-infected cells superinfected with an excess of RAV produced as many

TABLE 3

EFFECT OF X IRRADIATION ON FOCUS FORMATION BY CELLS DURING EARLY STAGES OF SOLITARY RSV INFECTION

Inoculum	Treatment	Days after infection 1	2	3	4	5
RSV (100 FFU)	Unirradiated	96*	460	1660	3300	6160
	Irradiated	0	4	14	100	238
RSV (100 FFU) plus RAV (10^7 infectious units)	Unirradiated	72	980	2280	7400	...
	Irradiated	60	616	2200	7300	...

* Calculated number of foci produced by plating all the cells from a single infected culture.
Cultures were infected with 100 FFU of RSV, or 100 FFU of RSV plus 10^7 infectious units of RAV. On each subsequent day the cells were suspended, an aliquot was X-irradiated with 5,000 *r*, and the unirradiated and irradiated samples were plated for focus formation on fresh chick embryo cell cultures.

foci after irradiation as before, indicating that irradiation did not suppress the initiation of foci by virus-producing cells. The results, therefore, show that the vast majority of cells infected with RSV alone fail to produce virus during the early stages of infection. The few foci produced after irradiation of the cells infected with RSV alone were probably due to unavoidable contamination by RAV during the various experimental manipulations.

Discussion.—The failure of the high titer strain of RSV to produce infectious virus in single infection may be compared to other systems in which deficiencies in the production of infectious virus have been found. Three such systems which have been subjected to critical analysis are the production of incomplete influenza virus in the Von Magnus phenomenon, the absence of virus-like materials in cells transformed by infection with polyoma virus, and the inability of defective bacteriophages to produce infectious virus in the absence of a helper virus.

The conditions for infecting cells so that they release incomplete influenza virus are the opposite of those required to produce NP Rous sarcoma cells. Cells which release incomplete influenza virus result only after multiple infection by influenza virus,[5] while NP cells can only be produced with low multiplicities of infection by RSV. This indicates a fundamental difference between influenza and RSV, since single influenza virus particles are fully capable of producing infective progeny, while single RSV particles are not.

In the case of polyoma virus, attempts to demonstrate the presence of virus-related material such as infectious DNA or virus protein in certain lines of transformed cells have failed.[6] When such cells are superinfected with another variant of polyoma virus, they produce only the superinfecting type, i.e., there is no indication of a helper virus action.[7] Therefore, the state of the polyoma virus, if it is present at all, in transformed cells is different from that of RSV in NP cells.

There are, however, striking similarities between the defective bacteriophages and RSV. Like RSV, the defective bacteriophages are unable to produce infectious progeny unless a helper virus is present.[8–11] Therefore, stocks of defective bacteriophage, like RSV, are mixtures which always contain helper virus. That the genome of the defective bacteriophage can multiply when it is established as prophage is shown by the fact that it is present in the progeny of a lysogenic bacterium. The genome of RSV also multiplies since a high proportion, if not all, of the NP cells produce infectious RSV when superinfected with RAV. Therefore, we conclude that the high titer strain of RSV is a defective virus analogous in some respects to the defective bacteriophages.

However, it is unwarranted to carry the analogy with defective bacteriophages

too far since fundamental differences are likely to be found. One difference already known is that RSV contains RNA as its genetic material and the defective bacteriophages contain DNA. Speculation about the precise nature of the defectiveness of RSV will be postponed until the results of current studies are available.

If it seems remarkable that the defectiveness of RSV has gone unrecognized for so long, it should be pointed out that the existence of the helper virus in the RSV stock was unsuspected until two years ago. The opportunity for its detection became available only with the advent of the interference test for avian leukosis viruses.[2] Even with full knowledge of the presence of RAV, rigorous precautions must be taken to prevent superinfection of transformed non virus-producing cells by RAV, since its concentration in the RSV stock is 10 times higher than RSV itself. Since production of RSV by transformed cells follows quickly upon RAV superinfection, previous growth curves obtained with this strain of RSV[12] must be reinterpreted in terms of the interaction with RAV.

Some confusion may arise from the fact that RAV, which plays an essential role in the production of RSV, is assayed by interference with RSV. RAV infection must precede RSV infection by several days for a highly effective interference to occur. There is no indication that the interference is reciprocal, since NP cells which have carried the RSV genome for many generations can readily be superinfected with RAV. The other avian leukosis viruses have a similar nonreciprocal interfering relationship to RSV.[2, 13] The fact that they do not appear to be defective suggests an association between their ability to produce infectious virus (i.e., to carry out a late stage of virus growth) and their ability to interfere (i.e., to suppress an early stage of RSV growth).

Thus far, clear evidence for the defectiveness of RSV has been provided only with the high titer strain. There are two findings which suggest that the standard strain of RSV is also defective. The first is the discovery by Temin that a large fraction of the chick embryo cells which are infected with this strain at a relatively low multiplicity and then are cloned on a feeder layer of mouse cells do not produce RSV unless another avian tumor virus is added.[14] When cloned on chicken cell feeder layers, however, the infected cells do produce RSV. The second is that a virus similar to RAV has been isolated from the standard strain (Shimizu, personal communication). A straightforward explanation for Temin's observations can be made, based on the fact that the avian tumor viruses grow poorly, if at all, on mouse cells while they flourish on chicken cells. A background of mouse feeder cells would minimize the spread of RAV to the RSV-infected chicken cells, while a background of chicken cells would facilitate such spread, and thereby elicit RSV production in the emerging clones.

It is of great importance to determine whether all strains of RSV are defective and are consequently always associated with helper viruses. If this is so, a rational explanation can be offered for such puzzling features of the biology of RSV as the failure to isolate infectious virus from tumors induced in chickens by low doses of RSV.[15] The absence of virus in such tumors cannot be explained on the same basis as the disappearance of virus from aging tumors induced with higher doses of RSV. The latter phenomenon occurs only in immunologically competent chickens and has been shown to be due to a cellular response by the host against the tumor cells.[16] The low dose noninfective tumors can be induced in baby chicks which are immu-

nologically incompetent, and the frequency of noninfective tumors does not depend on the age of the tumor. It seems likely such tumors are due to solitary infection and transformation of cells by defective RSV, which would be made possible by the low concentration of both RSV and RAV in the dilute inoculum.

Finally, attention should be drawn to the possible relationship between the defectiveness of RSV and its carcinogenic properties. The failure to perform the late virus functions could result in unrestrained production of the early virus products and thereby impair the regulatory functions of the cell.

Summary.—In an attempt to obtain the high titer strain of RSV free of RAV, isolated foci of transformed cells were produced by infecting chick embryo cultures with very high dilutions of the RSV stock. Agar, containing antibody to RAV, was added to the cultures to minimize the spread of RAV. The transformed cells were added to uninfected chick embryo cells and retained their altered morphology through repeated serial transfers. Most of the foci maintained in this way yielded no detectable virus. In every such case, however, RSV production could be elicited by adding RAV or any one of several other avian tumor viruses. A high proportion, if not all, of the transformed cells produced RSV upon superinfection with RAV, even when they had undergone well over 20 divisions in the absence of virus production.

A minority of the foci produced RSV spontaneously upon transfer and in every such case RAV was also produced. The proportion of foci producing RSV and RAV spontaneously was matched by the proportion of interfocal areas of normal cells which produced RAV alone. This indicated that RAV from the inoculum had infected cells at random and that the "spontaneous" production of RSV by transformed cells was due to the delayed spread of RAV following the removal of antiserum.

It is concluded that the high titer strain of RSV is defective since it cannot generate the production of new infectious virus unless a helper virus such as RAV is multiplying in the same cell. By contrast, however, the ability to cause the malignant transformation is expressed continuously, and consequently does not require the production of mature virus. Both the potential for virus production and the ability to transform cells are perpetuated in a cell line by hereditary transmission.

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